CLIMATE CHANGE & THE PHYSIOLOGY, ECOLOGY, & BEHAVIOR OF CORAL REEF ORGANISMS

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by

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CLIMATE CHANGE & THE PHYSIOLOGY, ECOLOGY, & BEHAVIOR OF CORAL REEF ORGANISMS

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I would like to dedicate my dissertation to my parents, Nancy J. Johnston & Ronald W. Johnston, without whom none of this would have been possible.

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LIST OF SYMBOLS AND ABBREVIATIONS

OA	Ocean acidification
L	Liter
mm	Millimeter
Cm	Centimeter
Ppt	Parts per thousand
H	Hours
Min	Minutes
mL	Milliliters
mL min^{-1}	milliliters/minute
s	Seconds
ANOVA	Analysis of variance
UV	Ultraviolet
pCO_2	Partial pressure of carbon dioxide
°C	Degrees Celsius
FL	Florida
Km	Kilometers
µm	Micrometer
LPH	Liters per hour
mL/min	Milliliters per minute
TA	Total alkalinity
cm^2	Centimeters squared
PAM	Pulse amplitude modulated

Fv/Fm	Photosynthetic efficiency
µatm	Microatmospheres
CO_2	Carbon dioxide
µmol kg^{-1}	Micromoles per kilogram
pCO_2	Partial pressure of carbon dioxide
HCO_3^-	bicarbonate
Ω_{ca}	Calcium saturation
Ω_{ar}	Aragonite saturation
SE	Standard error
IPCC	Intergovernmental Panel on Climate Change
RPM	Revolutions per minute
DNA	Deoxyribonucleic acid
rRNA	Ribonucleic acid
µL	Microliter
µM	Micromole
µg	Microgram
cells/g	Cells per gram
ESV	Exact sequence variants

SUMMARY

The magnitude of ocean acidification (OA) and warming predicted to occur within the next century could have significant negative effects for organisms that inhabit coral reefs. Our understanding of how these stressors will impact coral reef organisms is complicated by the diverse behavioral and ecological interactions that exist on these reefs. In a series of experiments, I explored interactions between coral reef organisms, evaluated how some of these interactions may be affected by OA and warming, and then studied how environment may shape an organism's response to a changing climate. First, through a sensory manipulated tank and a two-chamber choice flume, I demonstrated that anemonefish respond to both chemical and visual conspecific cues, but they require a combination of these two cues to correctly identify conspecifics. Given that previous research indicates that fish behavioral responses to chemical cues are altered under conditions of future OA, this inability to compensate for the loss of one cue through a second cue could affect their ability to acclimate as climate changes. Second, I found that the common Caribbean mounding coral *Porites astreoides* is unaffected by competition with *Montastraea cavernosa* and *Orbicella faveolata* under ambient environmental conditions but exhibits significant reductions in photosynthetic efficiency in areas of direct contact with *M. cavernosa* and *O. faveolata* under conditions of elevated CO_2 and temperature that are anticipated to occur by the year 2100. These results demonstrated that climate change can interact with competition to alter the rate and severity of coral-coral interactions on reefs of the future. Next, I compared the effects of OA and warming on the physiology of two congeneric coral species (*Oculina arbuscula* and *Oculina diffusa*)

representing temperate (*O. arbuscula*) and tropical (*O. diffusa*) environments and found that, although both corals were negatively impacted by ocean acidification and warming, the temperate coral was slightly more resistant to these stressors. This suggests that temperate species may not be as disadvantaged by climate change as one might expect and may not be easily displaced by more tropical species moving poleward as global oceans warm. Finally, I evaluated the effect of elevated temperature on the well-being of the temperate coral, *O. arbuscula* when collected from deeper more physically stable environments versus shallower more physically variable environments. I found that corals from both deep and shallow sites were negatively impacted by elevated temperature, but that corals from deeper sites were more strongly impacted. These findings suggest that the physiologies, biotic interactions, and behaviors of reef organisms may all be affected by climate change and that outcomes of these interactions may not be simple to predict as global oceans warm and acidify and as tropical organisms shift poleward and intermix with temperate species to form novel communities.

CHAPTER 1: ANEMONEFISHES RELY ON VISUAL AND CHEMICAL CUES TO CORRECTLY IDENTIFY CONSPECIFICS

Johnston NK, Dixson DL. 2017. Anemonefishes rely on visual and chemical cues to correctly identify conspecifics. Coral Reefs. 36(3):903-912.

1.1 Abstract

Organisms rely on sensory cues to interpret their environment and make important life-history decisions. Accurate recognition is of particular importance in diverse reef environments. Most evidence on the use of sensory cues focuses on those used in predator avoidance or habitat recognition, with little information on their role in conspecific recognition. Yet conspecific recognition is essential for life-history decisions including settlement, mate choice, and dominance interactions. Using a sensory manipulated tank and a two-chamber choice flume, anemonefish conspecific response was measured in the presence and absence of chemical and/or visual cues. Experiments were then repeated in the presence or absence of two heterospecific species to evaluate whether a heterospecific fish altered the conspecific response. Anemonefishes responded to both the visual and chemical cues of conspecifics but relied on the combination of the two cues to recognize conspecifics inside the sensory manipulated tank. These results contrast previous studies focusing on predator detection where anemonefishes were found to compensate for the loss of one sensory cue (chemical) by utilizing a second cue (visual). This lack of sensory compensation may impact the ability of anemonefishes to acclimate to changing reef environments in the future.

1.2 Introduction

Organisms throughout marine and terrestrial ecosystems rely on a combination of sensory cues to interpret and respond to their environments. Chemical, visual, and auditory cues are fundamental to most important life-history decisions, including predator avoidance (Stauffer and Semlitsch 1993; Bouwma and Hazlett 2001; McCormick and Manassa 2008), habitat selection (Lecchini et al. 2005), and mate recognition (Candolin 2003). The ability to respond to sensory information is critical to survival in the complex, diverse, and dynamic environment of a coral reef. The study of sensory perception in coral-reef organisms, particularly reef fish, is a vital part of reef research, and experiments demonstrate that reef fishes rely on a combination of sensory cues to avoid predation (Larson and McCormick 2005; Karplus et al. 2006; McCormick and Manassa 2008; Dixson et al. 2010) and identify appropriate habitat (Dixson et al. 2008; Leis et al. 2011).

While a large body of research highlights the role of sensory perception in life-history decisions of reef fishes, much less information is known about the specific cues to which organisms respond. For example, it is clear that reef fish exhibit reduced activity and increased bobbing behavior in response to a predator (McCormick and Manassa 2008), but the cue to which they are responding is less clear. Some reef fishes respond specifically to the predator's diet rather than the predator themselves (Dixson et al. 2012), while others alter their behavior in response to damaged conspecifics (Schoeppner and Relyea 2005). Conspecific-generated chemical cues are also believed to be important in habitat recognition (Sweatman 1988; Lecchini et al. 2005). More than 25% of the ocean's inhabitants are found on coral reefs, which constitute an area less than 0.01% of the ocean's benthic area; therefore, closely related species are often found near one another. The ability

to correctly identify and respond to conspecific cues likely provides more information about the species-specific safety and habitability of a specific location than generalized environmental cues while aiding in the recognition of potential mates. It is even more likely that reef fishes would rely on a combination of sensory cues in conspecific recognition.

It has been argued that reef-fish larvae rely on a hierarchy of sensory cues from conspecifics and the environment when making settlement decisions (Kingsford et al. 2002). This has been supported by research on the French grunt, *Haemulon favolineatum,* which demonstrated that post-larva *H. favolineatum* rely on a combination of visual and chemical cues from conspecifics and the environment for orientation (Huijibers et al. 2012). While there are no direct coral-reef studies evaluating the relative importance of different sensory cues in conspecific recognition, freshwater research supports the hypothesis that fish rely on a combination of visual and chemical cues to differentiate between conspecifics and heterospecifics (de Caprona and Ryan 1990). Experiments assessing the role of multisensory perception of conspecifics in reef fishes would enhance our understanding of how individuals make important life-history decisions such as mate and habitat recognition. They would also lay the foundation for future studies to evaluate the relative importance of conspecific sensory cues on reef-fish behavior over a variety of spatial and temporal ranges.

Anemonefish provide an ideal model for studying the role of multisensory species recognition in coral-reef fishes. Anemonefish (Pomacentridae) comprise 30 species; all exist in the Indo-Pacific and are characterized by their obligate relationship with ten species of host sea anemones (Fautin and Allen 1997; Ollerton et al. 2007; Allen et al. 2008, 2010; Litsios et al. 2014). Anemonefish are commonly found either in pairs or groups within a

host anemone or a colony of host anemones (Fautin 1991). Host anemone selection occurs when larval anemonefish transition from the pelagic environment to the benthos at the conclusion of their larval phase (Elliott et al. 1995; Arvedlund et al. 1999). While multiple species of fish use the same host sea anemone species, anemonefish species rarely co-exist in the same anemone (Fautin 1991; Elliott and Mariscal 2001; but see Camp et al. 2016). This failure to cohabit is likely due to the strong territorial behavior exhibited by juvenile and adult anemonefish occupying an anemone (Ross 1978), but it is also possible that anemonefish can differentiate conspecifics from less closely related species through sensory cues. Anemonefish are known to use chemical and auditory cues when making life-history decisions (Dixson et al. 2011; Simpson et al. 2011), and sensory perception is likely important in conspecific recognition. While post-settlement movement is not considered common, post-settlement inter-colony migration of juveniles (Ross 1978) and forcible evictions of subordinate individuals (Buston 2003) has been suggested. Additionally, hybrid anemonefish species have been found in natural settings (van der Meer et al.2012), suggesting that post-settlement interspecific interactions occur.

In this study, a series of choice trials were used to ascertain how cue availability impacted conspecific recognition in early juveniles of three anemonefish species, *Amphiprion clarkii, A. ocellaris,* and *Premnas biaculeatus*. Experiments were conducted in the presence and absence of another species. During each trial, an individual was evaluated for the amount of time spent near a conspecific and the activity level displayed. Furthermore, this study assessed the use of multiple sensory cues and the importance of each sensory signal independently and in combination. This experimental design tested the hypothesis that the loss of one sensory cue (either visual or chemical) would decrease the

ability of an individual to recognize a conspecific, thus reducing the time spent near a conspecific and increasing the activity level. Due to the known reliance of anemonefish on chemical signals for habitat selection, chemical cues were further isolated and conspecific detection was tested using a two-channel choice flume. Taken together, these hypotheses evaluated how anemonefishes use multisensory cues to recognize conspecifics when making important life-history decisions.

1.3 Methods

Species

Three species of anemonefish (*A. ocellaris, A. clarkii,* and *P. biaculeatus*) were tested for their response towards conspecific cues. Species were chosen based on known host anemone associations. *Amphiprion clarkii* is capable of forming a symbiotic relationship with all available anemone species, including those used by *A. ocellaris* and *P. biaculeatus.* However, *A. ocellaris* and *P. biaculeatus* are more specialized and do not inhabit the same anemones. Heterospecific recognition and avoidance may be more important in species that geographically overlap and share host sea anemone species. The ability of an individual to recognize species with similar host anemone associations was experimentally evaluated by comparing inter- and intraspecific recognition among the three focal species.

Habitat-naïve juveniles were sourced from a commercial breeder (Sustainable Aquatics, Jefferson City, TN, USA) and species were housed in individual 10-L aquaria within a closed 382-L recirculating system. Each recirculating system contained 24 10-L aquaria. No more than five conspecifics were housed within the same aquarium within the

recirculating system. Individuals that were housed within the same aquarium were not used in the same test, to ensure aquaria did not become a confounding factor. The three anemonefish species were housed in the same recirculating system for logistical reasons. Any chemical cue buildup within the system was prevented through daily water changes. This design kept laboratory-bred fish isolated from other species during the development period and permitted testing of innate species recognition. All fish were fed daily with a 0.8-mm pellet dry hatchery diet (Sustainable Aquatics, Tennessee USA) and were monitored for health and wellbeing in accordance with Institutional Animal Care and Use Committee policies.

Experimental set-up

Experiments took place over the course of two weeks when juveniles (2–4 cm standard length (SL); 3–4 months post hatching) were run in an 8.7 L closed tank filled with 6 L of artificial seawater (26.5–27.0 °C, 28–30 ppm salinity, Instant Ocean, Blacksburg, Virginia, USA). Before beginning trials, the experimental tank was divided into three compartments: two smaller outer compartments and one large inner compartment (Fig. 1.1).

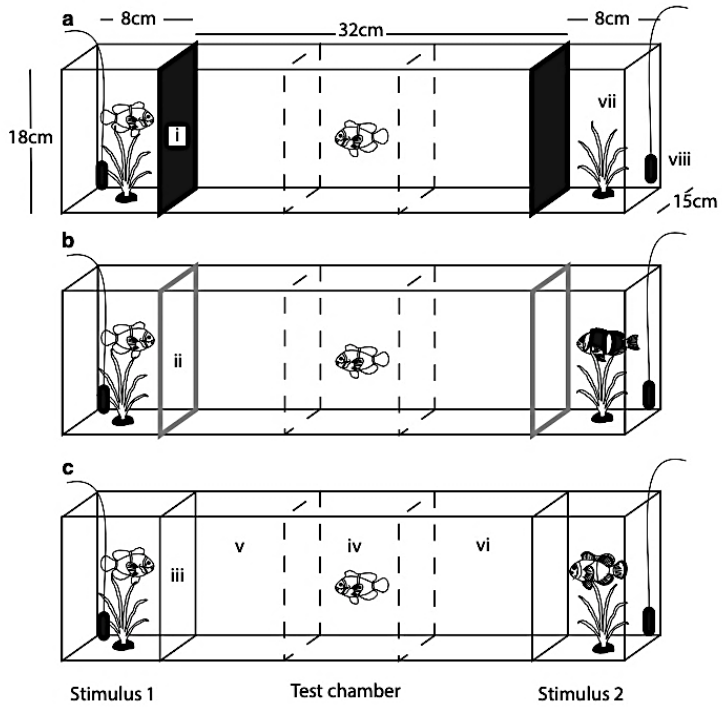

Figure 1.1 Diagram of experiment chamber used to evaluate sensory cue use in conspecific recognition. Sensory signals were manipulated by altering the barriers to the stimulus chambers. a removal of visual cues was created with unsealed opaque glass partitions (i); b removal of olfactory cues was created with sealed clear glass partitions (ii), and c all sensory cues provided was created with unsealed clear glass partitions (iii). *Dashed lines* represent the clear glass panes used during the habituation period. *Dashed lines* are also used for lines crossed measurements and delineating compartments in the test arena: no choice/habituation area (iv), near conspecific area (v) and other – either heterospecific or empty (vi). All sensory variables were cross-factored by species. In this diagram *Premnas biaculeatus* is used as an example. a conspecific vs. empty; b conspecific vs heterospecific (*Amphiprion clarkii*); and c conspecific vs. heterospecific (*A. ocellaris*). (vii) artificial saltwater aquarium plant, (viii) airstone and airline

The smaller outer compartments were separated from the larger inner compartment using glass dividers. Identical glass dividers were later used in sensory manipulations (see below). Anemonefish demonstrate hierarchical behavior and habitat use (Gainsford et al. 2015). To avoid any confounding factors involving hierarchical behavior, care was taken to use individuals of similar sizes. At the start of each trial, two conspecifics were chosen from a random pool of individuals within a size class. Individuals that were not active

participants in the experiment remained in the recirculating system. Individuals were placed in the two outer compartment treatments (0-1 individuals per outer compartment) before the test fish was placed in the center compartment. The species in the outer compartments was manipulated to evaluate whether anemonefish use sensory cues to differentiate between conspecifics and heterospecifics. The outer compartments held a conspecific, a heterospecific or no fish (empty) depending on the experimental trial. An artificial saltwater aquarium plant was also placed in the outer compartments for shelter and to minimize stress. Additionally, one airstone was placed in the back of each smaller compartment to keep the water oxygenated and facilitate water movement. The movement of water among the compartments was confirmed using a series of dye tests prior to each water change (Figure A1). Three paired combinations of experimental manipulations were conducted: (1) focal fish species compared to empty; (2) focal fish species compared to heterospecific species one; and (3) focal fish species compared to heterospecific species two.

Sensory cues emitted from the outer compartment were manipulated to determine the effects of the loss of either olfactory or visual cues on conspecific recognition by adjusting the seal and clarity of the panes separating the outer compartment from the experimental portion of the tank. Visual cues were blocked by blackening the glass panes using electrical tape. The panes were soaked in artificial saltwater (36 ppt) for 48 h to minimize the possibility that chemicals leaching from the electrical tape might affect the experiment. Electrical tape was chosen over black glass or acrylic to use the glass panes designed specifically to fit the experimental aquarium. Chemical cues were blocked by sealing the gap between the glass panes and the tank using aquarium silicone. Aquarium

silicone was allowed to dry for 24 h and the experimental tank was soaked in artificial saltwater (36 ppt) for an additional 48 h to minimize any effect of silicone. Preliminary dye tests were conducted to ensure sealed panes eliminated water movement from the outer compartment to the test compartment and, when panes were unsealed, water containing chemical stimuli moved into the test compartment without mixing (Figure A1). Dye tests confirmed that water reached the center, and that while some mixing did occur, a gradient was maintained. Care was taken to use the same amount of either electrical tape or silicone for the glass panes of both outer compartments. Thus, any effect of either treatment (electrical tape or silicone) would be present on both sides of the tank and should impact the experiment equally. Using these tank manipulation methods, the following trials were conducted: (1) all sensory cues—a clear, unsealed glass pane; (2) chemical cues removed—a clear sealed glass pane; and (3) visual cues removed—a darkened unsealed glass pane. No experimental manipulation was conducted to isolate auditory cues due to logistical constraints. Sensory and species treatments were fully crossed for each species of anemonefish tested. Tank water was mixed between trials and replaced after five trials. This method ensured consistent temperature and salinity while also evenly distributing the potential buildup of chemical cues given limits on availability of artificial seawater.

All experiments were run in two tanks. Experiments were first performed in the presence of visual and chemical cues. Outer glass panes were then sealed with electrical tape and experiments were run in the absence of visual cues. Electrical tape was removed, the glass panes were sealed inside the tanks with silicone, and the effect of the absence of olfactory cues was evaluated. Focal fish were tested for each paired species combination (conspecifics, heterospecific, or empty) within each sensory manipulation experiment

before moving on to the next sensory manipulation. Fifteen individuals of each species were chosen for each experiment from a pool of 45–50 individuals. While this did necessitate repeating individuals from one experimental treatment to another, effort was taken to ensure the same individuals were not repeated for all experiments in an effort to randomize any effect of learned behavior.

Measuring conspecific response

At the beginning of each experiment, the conspecific treatments for the outer compartment were created. At the same time the test fish was placed in the central compartment of the tank for a 10-min habituation period. During this time, the large compartment was subdivided into three equal-sized compartments using two additional clear panes of glass. These glass panes confined the test individual to the center of the test arena. This allowed the available sensory cues from the outer compartments to reach the test fish while it habituated to the tank environment (Fig. 1.1). At the end of the10-min habituation period, a 5-min test period began. Scoring commenced after the inner panes restricting the movement of the test fish had been carefully removed with minimal water disturbance and the fish was able to move throughout the entire test compartment. During the test period, the fish was continuously monitored over the course of 5 min for its location inside the tank and the length of time it stayed at that location. Locations were recorded as (1) the side closest to the conspecific, (2) the side farthest from the conspecific, or (3) the center compartment (serving as a “no choice” area). Using a stopwatch, the number of seconds spent in each location was recorded each time the test fish moved from one location to another. A still observer, seated 2 m from the test aquarium carefully recorded data while creating minimal noise and movement that might affect the fish. At the

conclusion of the 5-min test period all fish were removed and another trial commenced. While this design precludes statistical differentiation among the three locations, it mirrors the design of many flume tests where an individual is counted as making a preference regardless of its proximity to the source of the cue as long as it is in that cue's stream. The location of the conspecific in the test compartment (either left or right) was alternated every three trials to minimize any effects of side preference on results.

Activity level

Activity level was measured in addition to the time spent in each section of the tank by noting the number of times the fish crossed between the three areas of the large test compartment (side by conspecific, center, side away from conspecific) during the 5-min test period. At the end of the test, the total number of times the two centers were crossed was recorded. This method evaluated how often the fish moved from the side closest to the conspecific to the side farthest from the conspecific, indicating level of interaction with the conspecific.

Two-channel choice flume tests

A two-channel choice flume was used to further isolate the importance of chemical cues in conspecific recognition (flume dimensions: 14 cm long × 4 cm wide × 4 cm deep; working volume 308 mL). Here, conspecific treated seawater, heterospecific treated seawater or untreated seawater were evaluated using a pairwise experimental design. Following the methods outlined by Gerlach et al. (2007), a constant gravity-driven flow of 100 mL min^{-1} per channel was supplied from two 10-L header tanks that contained conspecific, heterospecific chemical cues or untreated seawater, and was maintained using

flow meters. This flow rate ensured that fish were not struggling to maintain their desired location within the flume, and therefore were able to display chemical preferences. Dye tests were done at each water change to ensure the two channels had parallel water flow with no areas of turbulence or eddies.

An individual fish was released in the downstream end of the flume where it was free to move to either side or swim towards the preferred water source. Each trial consisted of a 2-min habituation period, followed by a 2-min testing period. During the test period, the fish's position (left or right side of the test chamber) was recorded at 5-s intervals. A 1-min rest period was provided; during this time the water sources were switched from one side to the other, to rule out a side preference. The 2-min habituation period and 2-min testing period were then repeated. Chemical cues were created by soaking five juveniles (either conspecific or heterospecific) in 10 L of still seawater for 2 h. After 2 h, fish were removed and the water was poured carefully into the header tanks for the choice flume experiment. An additional supply of cue water was created using the same methods with different individuals; this was used to replace the treatment water in the flume when it had run out. A minimum of 20 individuals per species were tested per trial and fish were tested only once per trial. All chemical choice trials were run blind to the observer.

Statistical analysis

Three-way ANOVAs were run to assess the effects of sensory information (vision, olfaction, or vision and olfaction), tank treatment (empty, species 1, species 2), and focal fish species (*A. ocellaris, A. clarkii*, *P. biaculeatus*) on: (1) the proportion of time the focal species spent near the conspecific, and (2) the activity level as determined by numbers of

lines crossed. Sensory information and tank treatment were nested within focal species for both analyses. Tukey's post hoc tests were used to identify significant differences among treatments.

Flume data was assessed through Kolmogorov–Simonov tests comparing the proportion of time that individuals spent in the stream of water containing the olfactory cue compared to the proportion of time that individuals spent on one side of the chamber when no cue was present (blank trial: seawater compared to seawater).

1.4 Results

Measuring conspecific response

There was no effect of the identity of the focal species on the focal species' response to the presence of a conspecific ($F_{2,26} = 1.247$, $p = 0.289$). However, there was a significant effect of cue availability (Fig. 1.2) and the presence of another species (Fig. 1.3) on conspecific response. Regardless of the identity of the focal species, the loss of olfactory cues resulted in the test individual spending significantly less time on the conspecific side of the test arena (Fig. 1.2). The presence of a heterospecific species on the opposite side of the tank also affected the response of a test individual to the conspecific ($F_{6,26} = 2.9471$, $p = 0.008$). In most treatments, an individual spent approximately 50% of their time in the location closest to the conspecific (as opposed to either neutrally in the middle or on the opposite side of the tank). When *A. ocellaris* was the focal species, however, the presence of *P. biaculeatus* on the opposite side of the tank resulted in less time spent on the conspecific side than when no heterospecific was present (Fig. 1.3).

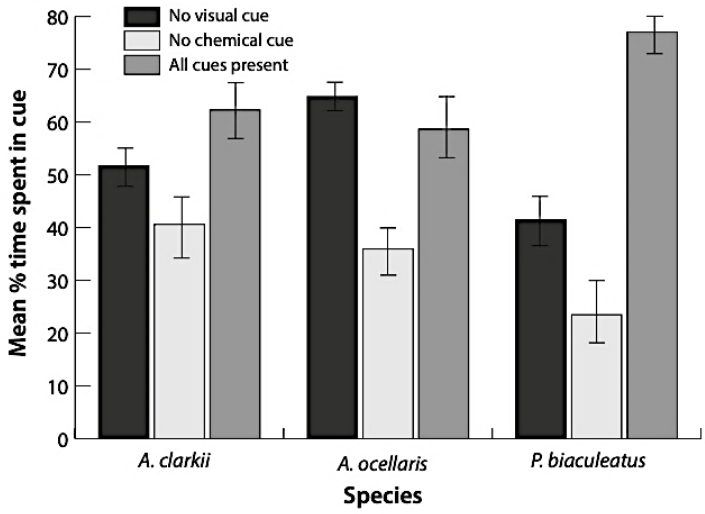

Figure 1.2 The mean percent time spent in conspecific cue across heterospecific treatments for each focal species. Error bars represent standard error.

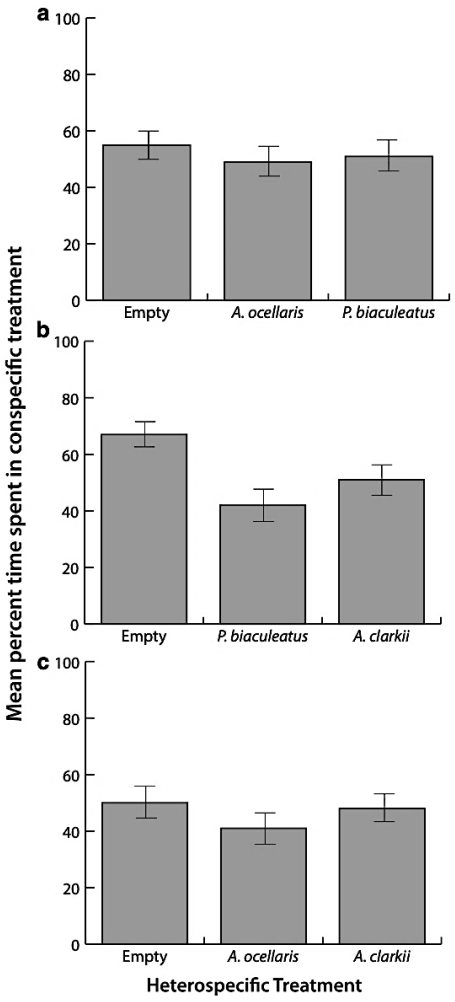

Figure 1.3: The mean percent time spent in the conspecific cue for each heterospecific treatment when a) *Amphiprion clarkii* b) *A. ocellaris* and c) *Premnas biaculeatus* is the focal species. Error bars represent standard error.

Activity level

Overall activity levels did not significantly among between anemonefish species ($F_{2,26}$ = 2.1724, p = 0.1153). Rather, the absence of a sensory cue and the presence of a heterospecific altered activity levels of all three species (sensory cue: $F_{6,26}$ = 29.738, p < 0.0001; heterospecific treatment: $F_{6,26}$ = 3.460, p = 0.002; sensory cue x heterospecific treatment: $F_{12,26}$ = 2.781, p = 0.001). Activity levels were approximately three (*A. ocellaris*)

to six (*A. clarkii* and *P. biaculeatus*) times higher when visual cues were absent than when all cues were present (Fig. 1.4). For *A. clarkii* and *P. biaculeatus,* the increased activity level in the loss-of-vision treatments differed depending on the presence of a heterospecific on the other side of the tank (Fig. 1.5). In the absence of vision, *A. clarkii* exhibited the highest average activity levels in the presence of *P. biaculeatus* (Fig. 1.5a). This response was not mirrored by *P. biaculeatus,* which exhibited higher activity levels in the absence of heterospecifics than in the presence of either *A. clarkii* or *A. ocellaris* (Fig. 1.5c).

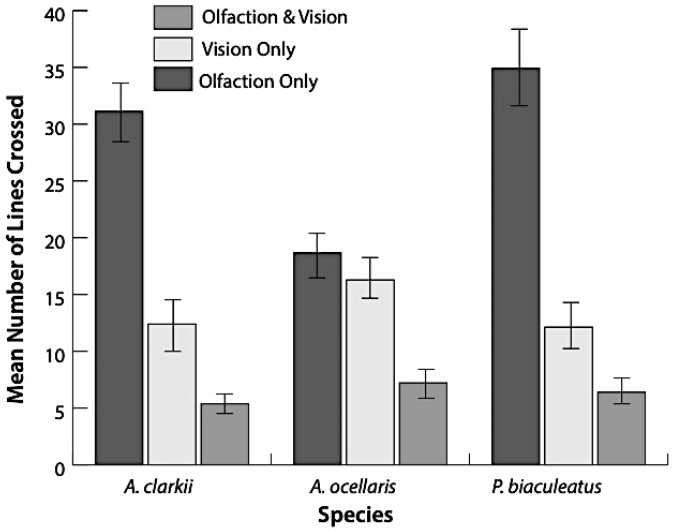

Figure 1.4: The mean number of lines crossed across heterospecific treatments for each focal species per five minute test period. Error bars represent standard error.

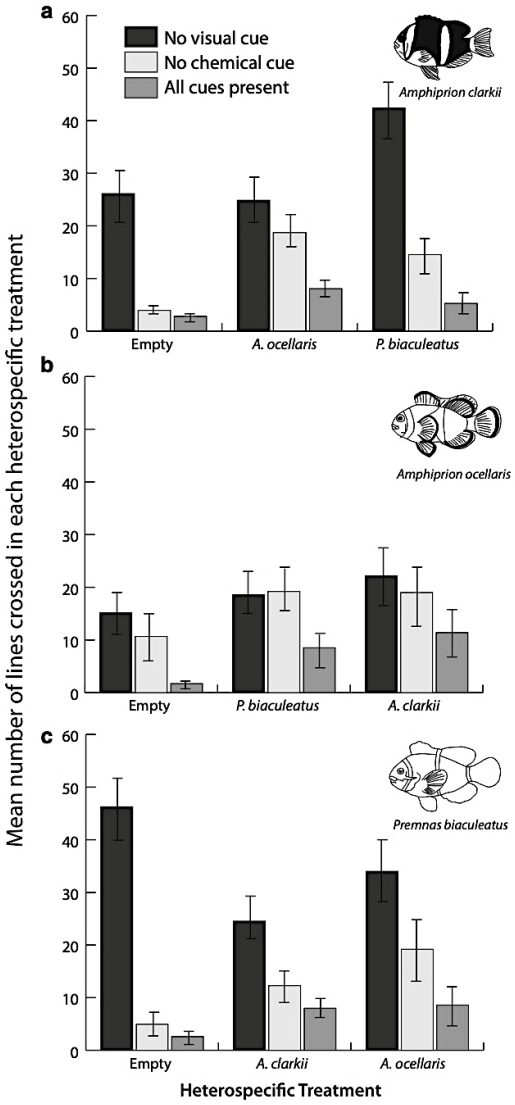

Figure 1.5: The mean number of lines crossed for each heterospecific treatment when a) *Amphiprion clarkii* b) *Amphiprion* ocellaris and c) *Premnas biaculeatus* is the focal species per five minute test period. Error bars represent standard error.

Two-channel choice flume tests

All anemonefish species tested displayed a strong attraction towards the chemical cues of the conspecific when tested against untreated seawater, with fish spending >85% of their time in this cue ($p < 0.001$ for all species). No preference or avoidance was shown towards the chemical cues of the heterospecific tested against seawater; fish spent an

average of 48% of their time in the heterospecific cue and 52% of their time in the untreated seawater ($p = 1.000$ for all species). However, when the chemical cues of the conspecific and heterospecific were simultaneously run, all species displayed strong preference for the chemical cues of the conspecific, spending more than 95% of their time in the water containing the conspecific chemical signal ($p < 0.0001$). No significant differences were detected in the strength of the preferences among the three species ($p = 1.000$).

1.5 Discussion

Our results demonstrate that anemonefish rely on a combination of sensory cues for conspecific recognition, and this recognition is affected by the presence of other species in the environment. The loss of either visual or chemical cues altered the behavior of the test fish by either increasing activity levels (loss of vision) or decreasing the time spent on the conspecific side of the tank (loss of olfaction). The increased activity levels exhibited during the loss of vision may be because the fish recognizes the chemical cue of a conspecific, but with visual confirmation lacking, the fish continues its search throughout the test arena. These results mirror freshwater results in which swordtail fish relied on a combination of visual and chemical cues in conspecific recognition (de Caprona and Ryan 1990), as well as results on the role of multisensory recognition in anemonefish for predator identification and habitat recognition (Manassa et al. 2013). Our results suggest that sensory signals provide the information necessary to distinguish between conspecifics and heterospecifics in these diverse areas.

The presence of a heterospecific on the opposite side of the tank influenced test fish behavior by increasing activity levels in the absence of vision and decreasing the time spent

on the conspecific side of the tank in the absence of olfaction. Interestingly, these differences in activity level depended on the identity of the heterospecific species but were unrelated to anemone host overlap between species. Flume data confirmed a difference in the chemical cues exuded by conspecifics and demonstrates that anemonefish are able to chemically differentiate between conspecifics and heterospecifics. Fish were attracted to water containing conspecific chemical cues, displayed no preference for water containing the chemical cues of heterospecific fish, and, when heterospecific and conspecific chemical cues were tested simultaneously, a strong preference for the conspecific was demonstrated by all species. A possible explanation for the differences in behavior in the tank experiments is that test fish recognized the conspecific, but that the presence of the heterospecific initiated territorial behavior. Anemonefish are well-known for defending their anemones (Ross 1978). Aggressive behavior was not directly measured in this study, but test fish were observed to attack the heterospecific side of the tank when a heterospecific fish was present. This behavior contrasted with test fishes' responses towards a conspecific; they were commonly observed near the glass pane without striking. Since anemonefish are commonly found in groups (Fautin and Allen 1997), it is possible that two conspecifics are attracted to one another but defend their territory from a heterospecific. It is impossible to draw conclusions on the type of behavior (attraction vs. aggression) in this experiment. There is sufficient evidence to indicate that the role of sensory cues in territorial aggression merits further evaluation.

To date, studies evaluating the role of multiple sensory signals in reef fish have focused primarily on their use in evaluating predation risk (e.g., McCormick and Manassa 2008; Manassa et al. 2013). While multiple cues are likely useful for habitat recognition

and settlement selection, sensory signals are typically evaluated either in complete isolation or in the field where all sensory cues are available (reviewed in Leis et al. 2011). This is the first study, to our knowledge, to focus solely on multisensory cue use for conspecific recognition. The results presented here support the general finding of the predation studies; reef fish use multiple sensory signals to process information. However, in the study presented here, anemonefish required both visual and chemical cues to identify a conspecific. This result contrasts with cue use for evaluating predation risk, where anemonefish and other reef fish require only one cue to alter their behavioral response (McCormick and Manassa 2008; Manassa et al. 2013). This is likely due to differences in the outcome of the response. By responding conservatively to a predator, a fish is more likely to avoid becoming prey and, thus, should respond at the mere hint of a predator. On the other hand, the situation modeled in this study is more analogous to behavior exhibited during recruitment and settlement. Anemonefish are extremely site attached, rarely moving from their host anemone once settlement has occurred (Fautin 1991; Buston 2003b). Thus, choosing a proper settlement site is necessary and, likely, would require multiple cues for confirmation.

It should be noted that the experimental design described here, while useful for manipulating visual and chemical cues for conspecific recognition, has two main limitations. First, it does not readily permit the isolation of auditory cues that some species of damselfish use in species recognition and dominance interactions (Parmentier et al. 2009; Colleye and Parmentier 2012). However, the goal of this experiment was to evaluate whether the loss of a single sensory cue affects conspecific recognition in the presence of other cues, and the results confirmed that it did. Second, these experiments were performed

in a laboratory rather than in the sea. For this reason, it is not possible to draw conclusions about the spatial scale over which sensory cues may be important (Kingsford et al. 2002; Leis et al. 2011). Nonetheless, these results suggest that fish may use cues serially for conspecific recognition. Anemonefish larvae develop in the pelagic environment before returning to the reef (Dixson et al. 2011). It has been hypothesized that during the recruitment period larvae first use chemical cues to locate reefs (Dixson et al. 2012), with visual cues becoming more important over smaller spatial scales. Recruitment occurs at night when light levels are at their lowest; chemical cues also extended farther distances than visual stimuli (Kingsford et al. 2002; Leis et al. 2011). The activity level differences between visual and chemical cues hint that chemical cues may serve as an initial indication of a nearby conspecific, while visual cues act as a final confirmation. The laboratory setting also did not permit the evaluation of visual communication using ultraviolet (UV) signals, which are important for some fish species (Siebeck et al. 2006). UV light should be an additional metric for consideration in future experiments. Nonetheless, its absence in this experiment does not detract from the main result that the loss of either chemical or visual cues affects conspecific recognition.

Chemical and visual cues are each important in anemonefish conspecific recognition; this has important implications for our understanding of how organisms use multisensory cues and the potential effects human activities may have on them. Ocean acidification alters chemoreception in damselfish (Munday et al. 2009; Dixson et al. 2010; Ferrari et al. 2011), impacting their ability to detect predators or locate suitable habitat (Dixson et al. 2010, Devine et al. 2012a,b). At the same time, pollutants such as humic acid reduce chemical mate recognition in freshwater fish (Fisher et al. 2006). It is possible that,

for some life-history decisions such as predator avoidance, individuals can compensate for the loss of one sensory cue using an alternative (Hartman and Abrahams 2000; Leahy et al. 2011; Lönnstedt et al. 2013). Our results indicate that some life-history decisions that rely on multiple sensory cues for confirmation may be affected by a changing world.

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CHAPTER 2: EFFECTS OF FUTURE CLIMATE ON CORAL-CORAL COMPETITION

Johnston NK, Campbell JE, Paul VJ, Hay ME (2019) Effects of climate change on coral-coral competition. Under Revision.

2.1 Abstract

As carbon dioxide levels increase, coral reefs and other marine systems will be affected by the joint stressors of ocean acidification (OA) and warming. The effects of these two factors on coral physiology are relatively well studied, but their impact on biotic interactions between corals are inadequately understood. While coral-coral interactions are less common on modern reefs, it is important to document the nature of these interactions on reefs with high coral coverage to better inform restoration strategies in the face of climate change. Using a mesocosm study, we evaluated whether the combined effects of ocean acidification and warming alter the competitive interactions between the common coral *Porites astreoides* and two other mounding corals (*Montastraea cavernosa* or *Orbicella faveolata*) common in the Caribbean. After 7 days of direct contact, *P. astreoides* suppressed the photosynthetic potential of *M. cavernosa* by 100% under both present (28.5°C and 401 pCO_2 and predicted future (30.5°C and 1054 pCO_2) conditions. In contrast, under present conditions *M. cavernosa* reduced the photosynthetic potential of *P. astreoides* by only 38%, while under future conditions reduction was 100%. A similar pattern occurred between *P. astreoides* and *O. faveolata* at day 7 post contact, but by day 14, each coral had reduced the photosynthetic potential of the other by 100% at the point of contact, and *O. faveolata* was generating larger lesions on *P. astreoides* than the reverse. In the absence of competition, OA and warming did not affect the photosynthetic potential

of any coral. These results suggest that OA and warming can alter the severity of initial coral-coral interactions, with potential cascading effects due to corals serving as foundation species on coral reefs.

2.2 Introduction

Though coral reefs cover <0.1% of the Earth's surface (Reaka-Kudla 1997), they are among Earth's most economically and ecologically valuable ecosystems (Barbier et al. 2011). In recent decades, coral reefs have been negatively impacted by a wide range of anthropogenic influences such as eutrophication, overfishing, and climate change (Hoegh-Guldberg et al. 2007, Hughes et al. 2010, Hughes et al. 2017) that have led to a 50-80% decline in global coral cover (Bruno et al. 2007, Jackson et al. 20). Ocean acidification (OA) and elevated temperatures are exacerbating this decline (Hughes et al. 2010, Hughes et al. 2017). Over the next 100 years, average sea surface temperatures are expected to increase 1-2 ℃, and ocean pH levels are predicted to decrease by 0.3-0.5 units (Caldeira & Wickett 2005, Collins et al. 2013). The 2016 and 2017 mass bleaching events brought on by higher than average water temperatures demonstrated that the effects of climate change are already dramatically and rapidly impacting coral reefs (Hughes et al. 2017) with a recent, five-fold increase in the frequency of mass bleaching events (Hughes et al. 2018). These rapid changes emphasize the need to better understand and predict the effects of climate change on these valued systems.

Given the foundational role of corals in tropical reef ecosystems, the effects of OA and/or ocean warming on coral physiology have been commonly investigated (Doney et al. 2009). OA and warming can independently reduce calcification and growth rates (Orr

et al. 2005, Cantin et al. 2010, De'ath et al. 2012), causes expulsion of dinoflagellate endosymbionts (Symbiodiniaceae) (Anthony et al. 2008), and, in worst cases, leads to coral death (Doney et al. 2012, Ainsworth et al. 2016, DeCarlo et al. 2017). At the same time, the magnitude of the physiological responses to OA and warming can vary among taxonomic groups, populations, and locations (Anthony et al. 2008, Pandolfi et al. 2011, Riegl et al. 2011, Kroeker et al. 2013, Harvey et al. 2013) with at least some corals withstanding some of the negative effects of warming and OA (McCulloch et al. 2012, Harvey et al. 2013). These complicated, context-dependent responses to OA and warming make predicting the potential outcomes of interactions between species difficult and highlight the need for direct investigations of how OA and warming impact coral-coral interactions (Kroeker et al. 2017).

Corals compete for space on coral reefs with many competitors including algae (McCook et al. 2001), sponges (Elliot et al. 2016, Chaves-Fonnegra et al. 2017), soft corals (Sammarco et al. 1985), ascidians (Bruno & Witman 1996), and other hard corals (Lang 1971,1973, Wellington 1980). Of these interactions, the effects of OA and warming on coral-coral interactions have rarely been investigated, likely due to the considerable decline in coral cover on many reefs over recent decades which has made coral-coral contact less common (Gardner et al. 2003, De'ath et al. 2012). However, regions or habitat patches with high coral cover can still be found across the globe (Guest et al. 2018). Moreover, given the right conditions, reefs have been shown to rapidly recover from low to high coral cover (Morgan et al. 2016, Edmunds 2018). These results demonstrate that in certain locations, given proper management strategies, coral-coral interactions are not uncommon.

Corals compete by using mesenterial filaments to digest neighboring corals' tissues (Lang 1971, 1973), and in some cases, can form sweeper tentacles in response to direct competition in order to increase their length of digestion (Chornesky 1983). Coral communities form competitive hierarchies, with some corals dominating others (Lang 1971,1973); however, these competitive outcomes can also reverse over longer periods of time (Wellington 1980, Chornesky 1989) and lead to changes in coral diversity (Connell 2004). A limited number of studies focused on understanding the effects of OA on coral-coral interactions have found mixed results. In some cases, OA affected coral growth (i.e. linear extension) without affecting competition (Evensen et al. 2015) or led to the early extrusion of mesenterial filaments (Evensen & Edmunds 2018). In another case, OA significantly impacted intraspecific competitive interactions in five species while leaving strong interspecific competitive interactions unaffected with a model suggesting cascading effects on competitive hierarchies (Horwitz et al. 2017). These studies indicate that the effects of OA on coral-coral competition are mixed and suggest the need for further study. Moreover, the simultaneous effects of increased carbon dioxide and temperature on coral-coral competition have rarely been evaluated.

This study assessed how OA and warming affect competition among common mounding corals in the Caribbean (*P. astreoides* versus *O. faveolata* and *M. cavernosa*). These species are among the most common corals in the Florida Keys, are considered to be relatively resistant to several common stresses (e.g. bleaching, predation - Burman et al. 2012), and may be the most common remaining competitors on modern, and likely future, Caribbean reefs. Variations in their responses to OA and warming may alter these coral-coral competitive interactions and create new competitive winners and losers on coral reefs.

2.3 Methods

Experiments assessing the combined effects of OA and warming on coral-coral competition were conducted at the Smithsonian Marine Station in Fort Pierce, Florida. Two experiments were run sequentially over a period of seven weeks, with the first experiment running 16 days between 2 and 18 October 2017 and the second experiment running for 23 days between 18 October and 9 November 2017. Three colonies of *M. cavernosa* and *O. faveolata,* and six colonies of *P. astreoides* (each 30-45 cm in diameter) were collected from the Florida Keys National Marine Sanctuary coral nursery in Key West, FL, maintained in raceways with running seawater for at least 2 months, and then used in the experiments outlined below.

Experiment One: Porites astreoides vs Montastraea cavernosa

After the initial two-month acclimation period, three colonies of *P. astreoides* and three of *M. cavernosa* were each divided into eight fragments (each fragment 7-10 cm). Coral fragments recovered in the raceways for 48 hours prior to being moved to 12 37-L aquaria designed to manipulate carbon dioxide levels and temperature individually in each tank. Seawater was collected from 0.4 km offshore from Fort Pierce, Florida, filtered (<10 μm), and water recirculated within each tank using a 473 LPH powerhead. Tanks were randomly assigned to either present (401 pCO_2 and 28.5 °C) or future (1054 pCO_2 and 30.5 °C) oceanic conditions (Table 2.1, n=6 per treatment). Present treatments are based on current conditions, while the future treatments were designed to mimic the predicted most extreme scenario by the IPCC (RCP 8.5) (Meinshausen et al. 2011) (Table 2.1). This design was chosen over a fully factorial experiment because temperature and OA are predicted to

both change over time, thus representing future conditions, and to allow for greater sample size and power to detect effects on coral-coral interactions. pH was continuously controlled and monitored using a pH stat computer (Aqua Medic) which bubbled 100% CO_2 into each tank (25 mL/min) as necessary to maintain treatment levels. Temperature was monitored and controlled using independent dual-stage digital controllers attached to water-jacketed heat exchangers. pH, temperature, and salinity were also externally monitored and verified one to two times daily using a ThermoFisher Orion Star pH meter (relative accuracy ±0.01 units) and a YSI temperature/salinity meter. Water changes (25%) occurred twice weekly with small additions of deionized (DI) water daily as needed to maintain salinity near 36ppt in both present and future treatments (Table 2.1). Total alkalinity was measured weekly via open-cell potentiometric titration. Carbonate parameters within each tank were calculated in the CO_2SYS program using measured parameters of pH, TA, temperature, and salinity, with the carbonate dissociation constants of Mehrbach et al. (1973) as refit by Dickson & Millero (1987).

Table 2.1 Average (±SE) calculated carbonate chemistry parameters from the measured parameters of pH, total alkalinity (TA), temperature, and salinity (n=6).

Experiment	Treatment	Salinity	Temp (°C)	TA (µmol kg^{-1})	Ph_{NBS}	pCO_2 (µatm)	CO_2 (µmol kg^{-1})	HCO_3^- (µmol kg^{-1})	Ω_{ca}	Ω_{ar}
P. astreoides vs. *M. cavernosa*	Present	36.18 ±0.15	28.60 ±0.07	2340.49 ±33.12	8.18 ±0.02	373.75 ±21.82	9.55 ±0.56	1639.80 ±90.40	5.68 ±0.40	3.80 ±0.26
	Future	35.62 ±0.22	30.24 ±0.09	2474.24 ±50.51	7.89 ±0.01	1083.4 ±52.61	26.68 ±1.21	2125.99 ±47.05	3.49 ±0.07	2.35 ±0.05
	p-values	p=0.032	p<0.001	p=0.094	p<0.001	p<0.001	p<0.001	p=0.001	p=0.001	p=0.001
P. astreoides vs. *O. faveolata*	Present	36.3 ±0.17	28.47 ±0.05	2137.73 ±23.87	8.17 ±0.01	460.70 ±14.93	11.93 ±0.40	1655.87 ±21.69	4.60 ±0.11	3.07 ±0.07
	Future	36.59 ±0.39	30.28 ±0.11	2352.72 ±25.56	7.86 ±0.01	1107.99 ± 47.53	27.31 ±1.12	2025.81 ±20.02	3.21 ±0.15	2.16 ±0.1
	p-values	p=0.66	p<0.001	p=0.002	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001

Coral fragments were randomly assigned to treatments and tanks, and two fragments from each species (*P. astreoides* and *M. cavernosa*) were positioned randomly in each tank a minimum of 5 cm from each other to prevent any interactions via mesenterial filaments or sweeper tentacles (Chornesky 1983) for a total of 24 fragments per species. Coral fragments acclimated to tank conditions for 7 days, after which, contact was initiated. One fragment of *M. cavernosa* was placed in direct contact with a fragment of *P. astreoides* in each tank, and this treatment was maintained for 7 days. The area of direct contact was 6-7 cm^2. In the direct contact area, living surfaces were placed in contact with polyps oriented toward the surface of the other coral. The remaining fragments of each coral (no-contact controls) in each tank were positioned 10 cm away from the contact corals and from each other.

Endosymbiont photosynthetic efficiency was monitored using PAM fluorometry to determine maximum quantum yield (Fv/Fm – a measure of photosynthetic efficiency) every other day over the course of the experiment. Corals were dark-adapted for one hour prior to taking PAM readings, and readings were taken between 1100 and 1300 hours each day. For corals that were in direct contact, one PAM reading was taken at the area of contact with the other species and a second PAM reading was taken approximately 3 cm away from the area of contact with the other coral species, but on this same fragment. For the "no-contact control" corals that were not in contact with the other coral species, three PAM readings were taken randomly over the coral to get a mean for that individual. When coral lesions formed (this happened only for corals in contact), PAM values thereafter increased to values outside of the average readings for no-contact corals in this experiment. This appeared to be due to rapid colonization of the exposed coral skeleton by diatoms,

cyanobacteria, or other epilithic algae (as also noted by McCook & Diaz-Pulido [23]). Areas with a visible lesion (confirmed with photos and a zero or near zero PAM reading prior to epilithic algal growth) were recorded as a "zero" PAM reading – even following colonization by epilithic algae.

The combined effects of OA and warming on competition were evaluated by comparing differences in endosymbiont photosynthetic efficiency between points of direct coral-coral contact and points on the same piece of coral that were 3 cm distant from contact with the other species and evaluating these patterns across the two environment scenarios (present vs future). The effects of competition and environment on coral health beyond the areas of direct contact were evaluated by comparing photosynthetic efficiency values taken from areas of the competing corals that were 3 cm away from the direct contact area and comparing these values to values from corals in the same tank but not in contact with a competitor (the "no-contact" controls).

Lesion presence and size were recorded through daily photos (including a scale) using a Nikon Coolpix W300 camera to evaluate the effects of competition and OA/warming on coral tissue health. Lesions formed first via visible discoloration of coral tissue followed by tissue mortality and tissue loss. Distinctions were made between areas of the lesion that were discolored (i.e. areas where the color differed from other parts considered healthy including exposed coral skeleton) and areas with exposed coral skeleton and tissue mortality. Thus, lesions were quantified as the combined size of the discolored area and the area with tissue mortality. The total area of tissue mortality was also quantified separately. After the first 2-3 days of lesion formation, lesion size stabilized, and the size

of the areas that were discolored and/or contained dead tissue were only evaluated at day 7 using ImageJ software.

Experiment Two: Porites astreoides vs Orbicella faveolata

Because coral competitive outcomes can change over time as sweeper tentacles form in response to direct competition (Wellington 1980, Chornesky 1989) and because we wanted to evaluate among-species differences in competitive outcomes, we conducted a follow-up experiment using *P. astreoides* and *O. faveolata*. This experiment mimicked the previous experiment's design with minor variations. Three colonies of each species acclimated in the original raceways for an additional three weeks while we conducted the first experiment. As with the first experiment, they were then cut into 8 fragments and acclimated for 48 hours before being transferred to the 12-tank system. After acclimating to the 12-tank system for 7 days, interspecific coral interactions were initiated and monitored over a 14-day period (7 days longer than the previous experiment) to determine whether the effects of future conditions altered the outcomes of coral-coral competition. Once again, daily photos recorded lesion presence and changes in size. These photos were analyzed at day 7 and at the conclusion on day 14. PAM readings were taken every other day to monitor coral health.

Statistical Analyses

All analyses were conducted in R version 3.4.3 using the car 2.1-5, lmPerm 2.1.0, and multcomp 1.0 packages (Fox & Weisberg 2019). Data were evaluated for normality and equality of variance prior to analyses. Lesion size data did not meet the assumptions of equality of variance, and transforming the data failed to meet these assumptions, so two-

way permutation ANOVAs were used to evaluate these data using climate (present vs. future) and contact area (contact vs no-contact) as fixed effects. The effect of individual tanks on the data was analyzed using a one-way ANOVA and, lacking significance, was removed from the analysis. All other analyses were conducted using two-way ANOVAs with climate and contact area as fixed effects. One-way ANOVAs using the Bonferonni correction evaluated differences among treatments. Contrasts of physical parameters (temperature, OA, salinity, etc.) between treatments were conducted by averaging the many measures for each tank across time into one mean for each tank (i.e., using tanks as independent replicates) and comparing the six present treatment tanks to the six future treatment tanks via t-tests.

2.4 Results

Elevated temperature and CO_2 significantly changed competitive interactions between *M. cavernosa* and *P. astreoides* (Fig 2.1A, climate x contact, p=0.027). Under present climate conditions, contact with *M. cavernosa* for 7 days reduced *P. astreoides* Fv/Fm by 37.5%, but under future temperature and OA, contact reduced Fv/Fm by 100%. For *M. cavernosa,* direct contact with *P. astreoides* reduced Fv/Fm at the point of contact by 100%, regardless of climate conditions (Fig 2.1B, climate x contact, p=0.643). Neither climate condition, nor competition, affected Fv/Fm in non-contact areas (Fig 2.1C&D). Temperature and OA also did not significantly impact the size of the lesion formed at the point of contact for either coral species (Fig 2.2).

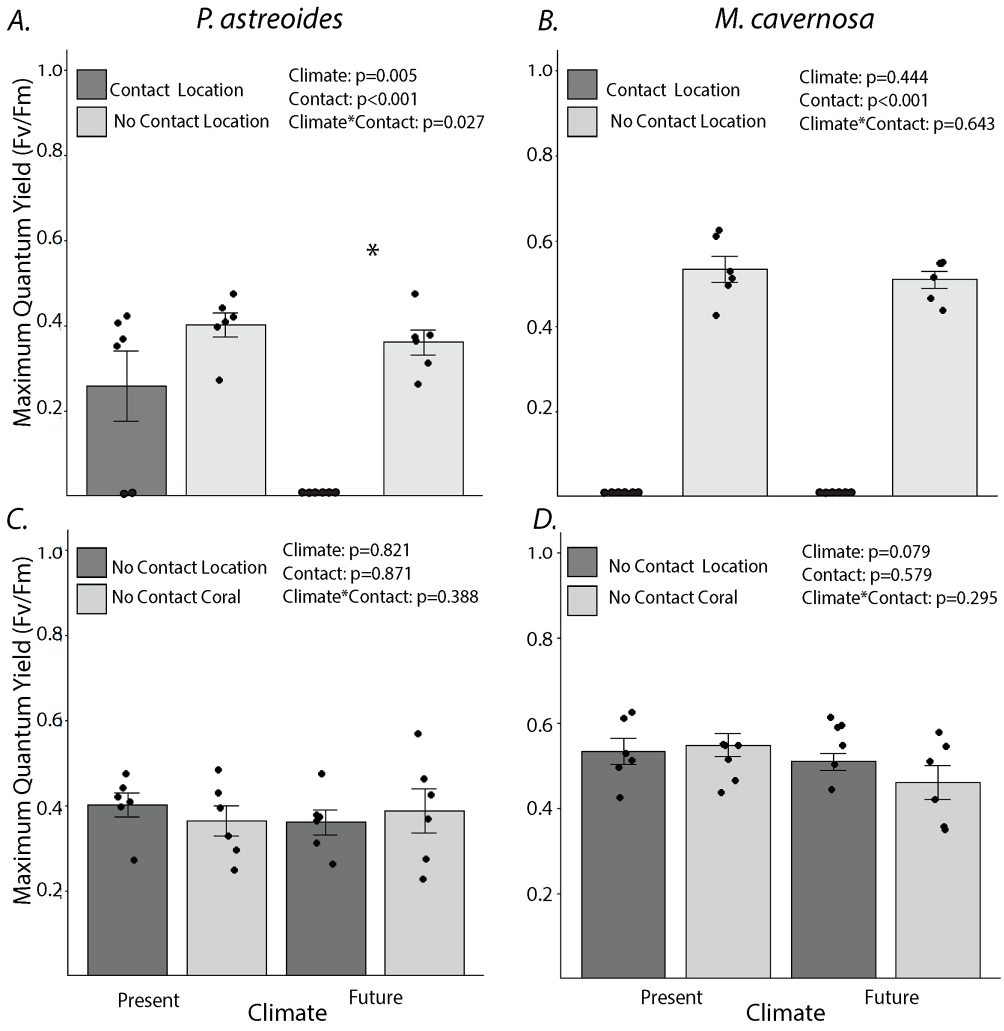

Figure 2.1 Effects of climate and contact with a competing coral on endosymbiont photosynthetic efficiency (Fv/Fm). (A and B) Maximum quantum yield values (means ± SE) on corals in areas that are (Contact Location) or are not (No Contact Location) in direct contact with another coral species. (C and D) Maximum quantum yield of locations 3 cm away from locations of direct contact for corals in contact with a competing coral (No Contact Location) versus for corals of that species that are not in contact with another competing coral species (No Contact Coral). Analyses by two-way ANOVA comparing contact and climate for each coral species. Dots show individual data points. N=6.

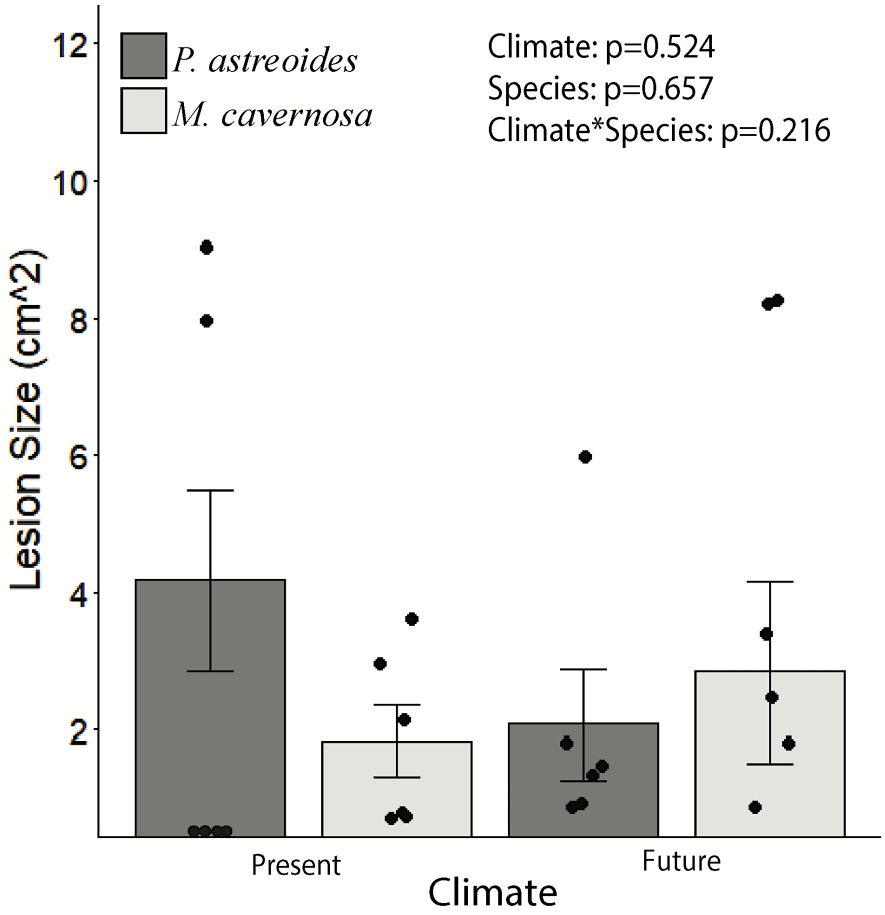

Figure 2.2 Lesion size (means ±SE) for each competing coral at the point of contact at Day 7. Analyzed with two-way permutational ANOVA comparing climate and species. Dots represent individual data points. N=6.

In the second experiment where *P. astreoides* and *O. faveolata* were in contact, areas of *P. astreoides* in contact with *O. faveolata* exhibited a 62.5% reduction in Fv/Fm under present conditions, but reductions of 100% under future OA and warming conditions at day 7 (Fig 2.3A, climate x contact , $p = 0.036$). For *O. faveolata*, contact with *P. astreoides* reduced Fv/Fm by 77-84% under both climate conditions (contact: $p<0.001$), and effects did not vary as a function of OA and temperature (Fig 2.3B; climate x contact: $p = 0.278$). At day 14, the patterns for *P. astreoides* were similar to those on day 7. Contact reduced *P. astreoides* Fv/Fm by 45-62% (contact: $p<0.001$), but there was no longer a

significant contact x climate interaction (Fig 2.3C, $p = 0.660$). For *O. faveolata*, patterns on day 7 persisted through day 14; competition reduced Fv/Fm by 84-100% (Fig 2.3D $p<0.001$), with no significant difference between climate conditions. As with the *P. astreoides-M. cavernosa* interaction, negative effects of competition on Fv/Fm were restricted to areas of direct contact. For *P. astreoides,* areas not in direct contact with *O. faveolata* exhibited significantly higher Fv/Fm than the "no-contact controls" on day 7, but by day 14, this pattern was no longer significant (Fig 2.4).

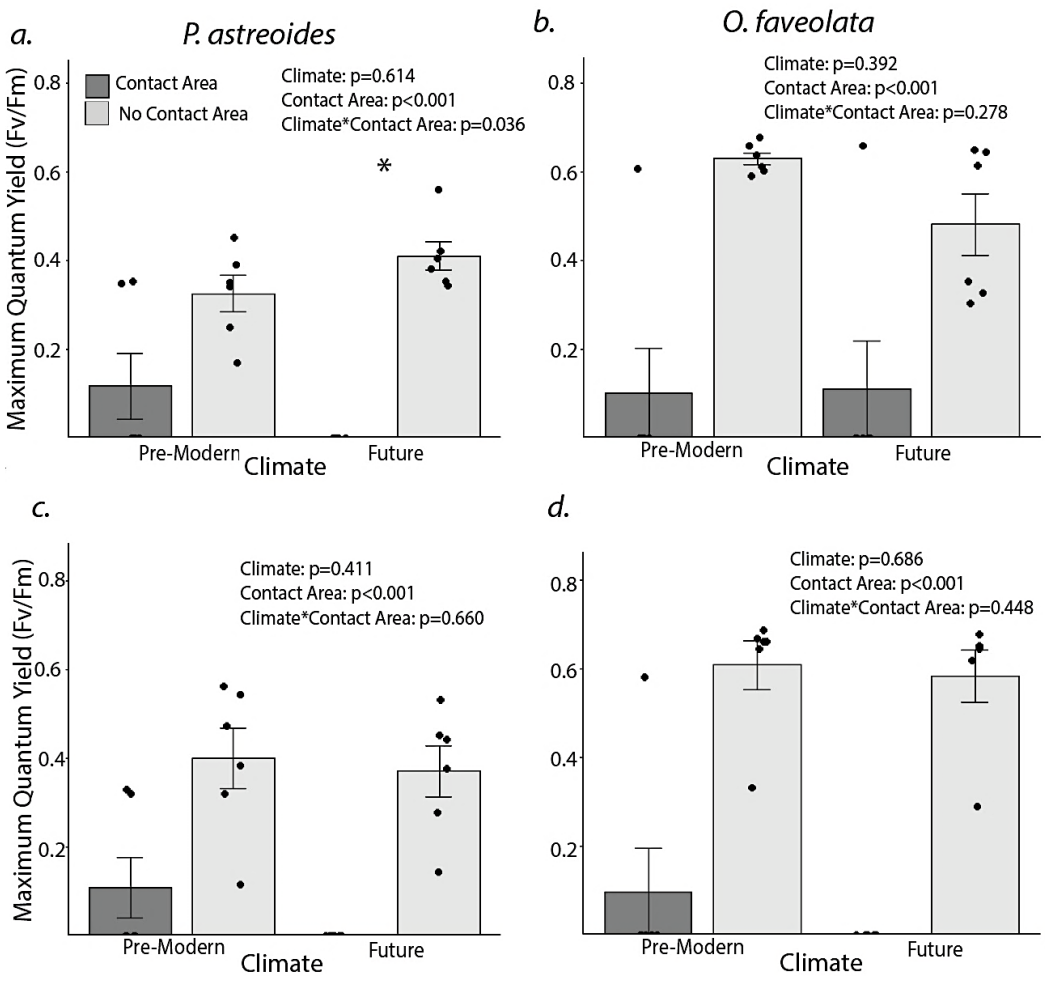

Figure 2.3 Effects of climate and contact with a competing coral on endosymbiont photosynthetic efficiency (Fv/Fm). Maximum quantum yield values (means ±SE) on corals that are (Contact Location) and are not (No Contact Location) in direct contact with another coral species at day 7 (A and B) and day 14 (C and D). Analyzed with two-way ANOVAs comparing contact area and climate. Dots represent individual data points. N=6.

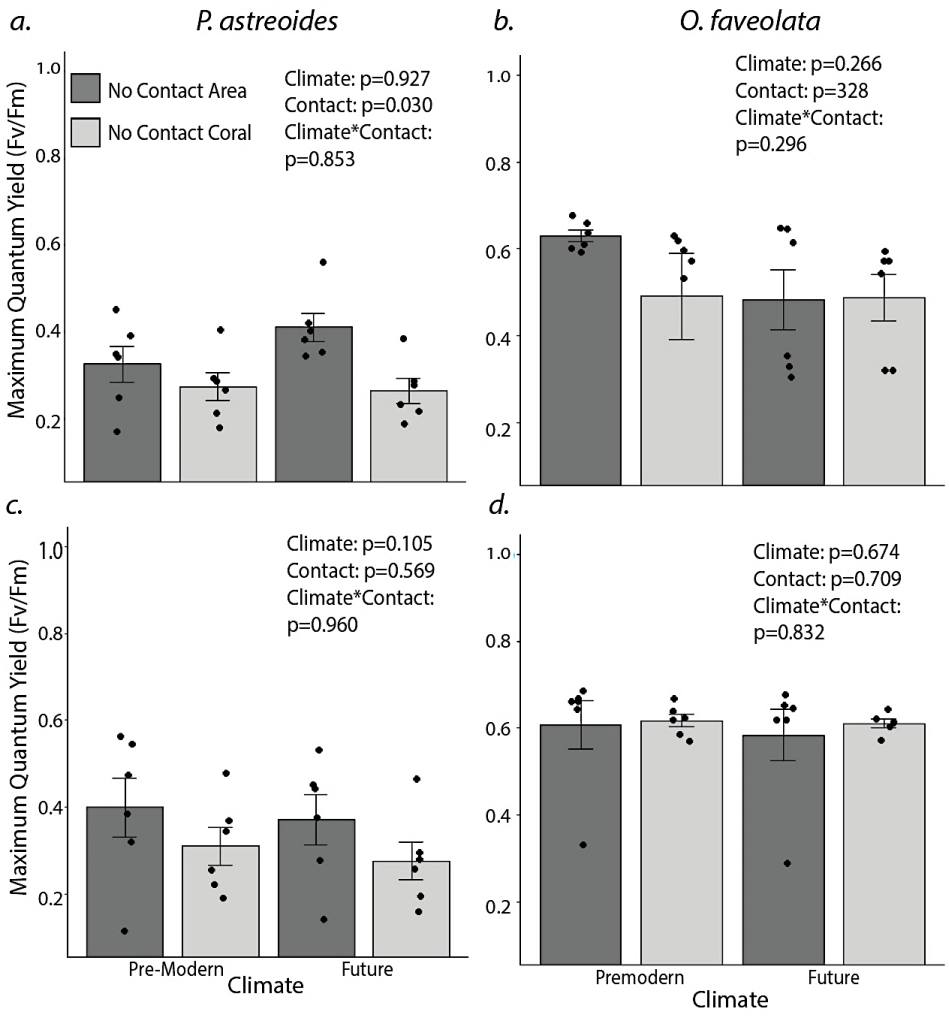

Figure 2.4 Effects of climate and contact on maximum quantum yield (means ±SE) Analyzed on coral locations that are 3 cm away from the point of direct contact with a competing coral (No Contact Location) versus corals that are not in contact with any other coral (No Contact Coral) at day 7 (A and B) and day 14 (C and D). Analyzed with two-way ANOVAS comparing contact and climate for each coral species. Dots indicate individual data points. N=6

As with the interaction between *P. astreoides* and *M. cavernosa,* competition between *P. astreoides* and *O. faveolata* was unaffected by temperature and OA (Fig 2.5). However, *P. astreoides* developed discoloration areas indicative of lesion formation that were 4x larger than those of *O. faveolata* at day 7 (Fig 2.5A, $p<0.001$) although there were no significant differences in the portions of the coral with bare skeleton (Fig2. 5C, $p=0.45$).

By day 14, the overall size of the discoloration area was no longer significantly different between the two coral species (Fig 5B, p=0.070); however, the area with complete loss of coral tissue was 133% larger on *P. astreoides* than *O. faveolata* (Fig 5D, p=0.048).

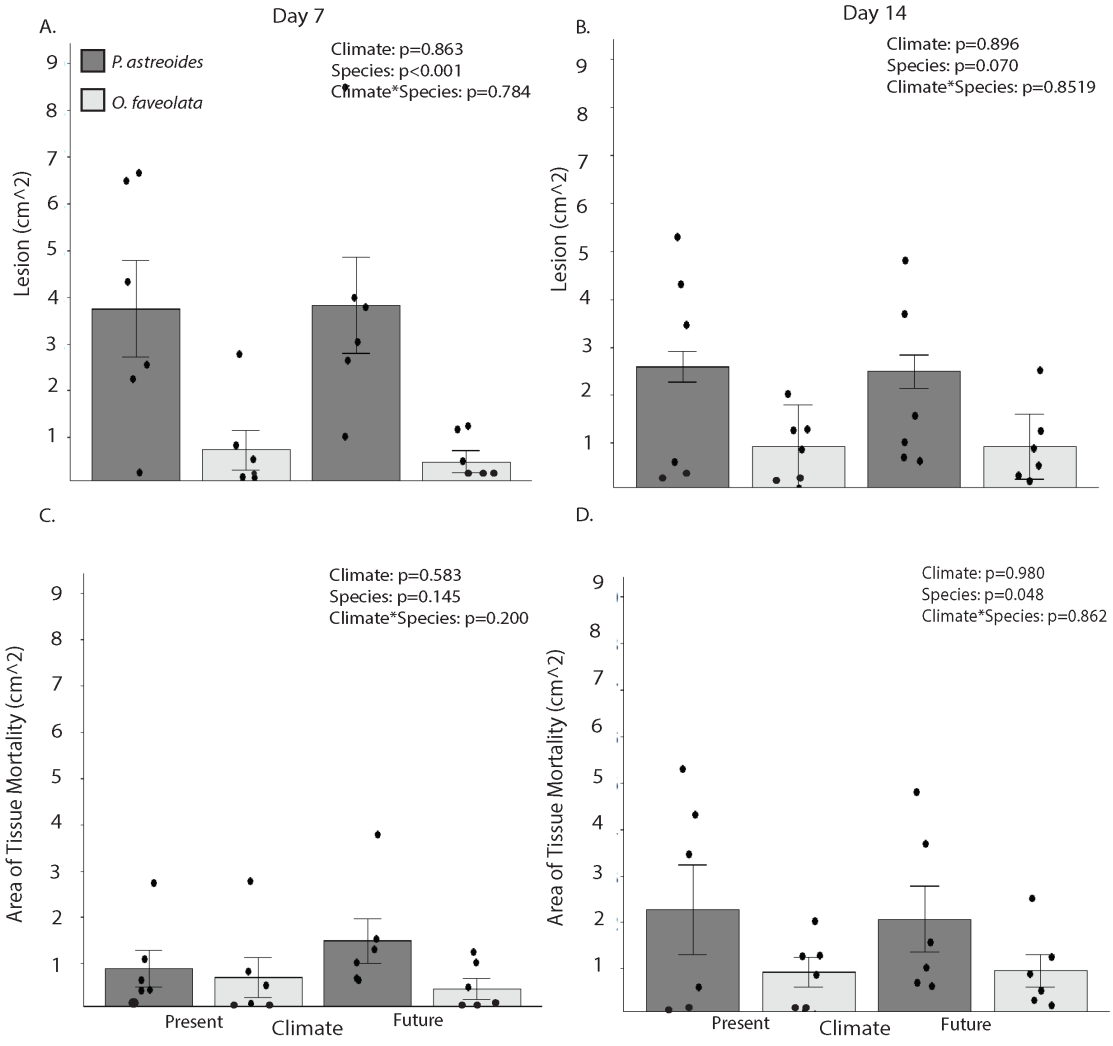

Figure 2.5 Lesion Size and Tissue Mortality at Day 7 and Day 14 Lesion size (Means ±SE) (A and B) and Area of Tissue mortality (Means ±SE) (C and D) for each coral in contact with the other at day 7 (A and C) and day 14 (B and D). Lesion area is the area of each coral that exhibited discolored coral tissue plus no coral tissue; Area of tissue mortality corresponds to areas of bare skeleton with no living tissue. Analyzed with two-way Permutational ANOVA comparing species and climate. Dots represent individual data points. N=6.

2.5 Discussion

Most experiments directly measuring coral-coral aggression were conducted decades ago when coral reefs were healthier, coral cover was greater, and coral-coral contact was more frequent. Those studies found that coral-coral interactions were hierarchical (Lang 1971, 1973), but could reverse over longer periods of contact (Wellington 1980). Here we show that climate change can impact the speed or severity of coral-coral competition for some species that are among the most common corals remaining on Caribbean reefs (Burman et al. 2012). Competition with *P. astreoides* negatively impacted *M. cavernosa* and *O. faveolata* in areas of direct contact regardless of climate treatment, however *P. astreoides* became more negatively impacted by contact with the other two corals in areas of direct contact under OA and warming.

Many experiments evaluating the effect of OA on coral reef competitive interactions have found that high CO_2 either enhances the susceptibility of the weaker competitor to the stronger competitor (e.g. high CO_2 enhancing algal competition over corals – Diaz-Pulido et al. 2011), reverses the competitive dynamics (e.g. in damselfish – McCormick et al. 2013), affects intraspecific competition more strongly than interspecific competition (Horwitz et al. 2017), or may not directly affect interspecific competition (Evensen & Edmunds 2018). Unlike these results, our data suggest that OA and warming increased the susceptibility of the stronger competitor, *P. astreoides,* to the weaker competitors, *M. cavernosa* and *O. faveolata,* in areas of direct contact at day 7 without impacting the negative effects that *P. astreoides* had on the weaker competitors. Outside of direct contact areas, none of the corals exhibited evidence of coral bleaching from competitive interactions or OA and temperature stress at day 7 (Fig 2.1C and 2.4A, B). The mechanism

altering the susceptibility of *P. astreoides* to competition with *O. faveolata* and *M. cavernosa* in areas of direct contact under OA and warming is unclear. The change in the susceptibility of *P. astreoides* to the other two corals could be due to oxidative stress, which is often a precursor to coral bleaching (Lesser 1997, Downs et al. 2002, Weis 2008), changes in the microbiome (Glasl et al. 2016), chemical defenses (Lages et al. 2006), faster mesenterial filament production (Evensen et al. 2018), or other mechanisms. Regardless of the factor, these results suggest that the OA and warming may alter coral-coral interactions.

While both experiments demonstrated an effect of OA and warming on coral-coral interactions at day 7, the second experiment ran for an extra 7 days to evaluate how these results held up over longer time scales. Other experiments have suggested that ecological interactions can outweigh the negative effects of OA and warming over longer time scales (Evensen et al. 2018)**.** As with these studies, OA and warming no longer significantly affected photosynthetic efficiency in areas of direct contact at day 14 (Fig 2.3). Increased variation of photosynthetic efficiencies in the no contact locations for both present and future treatments (Fig 2.3A vs Fig 2.3C) corresponded with an increase in the size of the tissue necrosis area from day 7 to day 14 for *P. astreoides* suggestive of continued competition (Figs 2.5C and 2.5D), and the results mirror other findings suggesting that OA sped up the production of mesenterial filaments while not affecting the final outcome of competition (Evensen et al. 2018).

Across both experiments, areas of discoloration and/or tissue mortality formed in response to competition, but the sizes of these areas were unaffected by OA and warming. In the first experiment, damage did not expand beyond the areas of direct contact, and there

was no significant difference in the size of the lesion area between the two coral species (Fig 2.2). In the second experiment, *P. astreoides* developed areas of discoloration twice the size of those formed by *O. faveolata* that extended beyond the areas of direct contact by day 7 (Fig 2.5). This may be evidence of the formation of sweeper tentacles known to form on some corals (Wellington 1980, Chornesky 1989), although the evidence of such formation was not specifically documented in this study. The area of discoloration on *P. astreoides* shrank 41% between days 7 and 14, leaving tissue mortality and bare skeleton in the areas of direct contact with recovered tissue outside areas of direct contact. Tissue mortality was likely due to direct interspecific aggression documented in other studies (Lang 1973). Some studies have suggested that environmental conditions can play a role in coral tissue recovery (Fisher et al. 2007, Denis et al. 2011), however they focused more on strong variations between environmental field conditions without explicitly evaluating specific conditions. In this study, there was no evidence that tissue recovery was dependent on present or future warming conditions supporting one other study that found tissue recovery of *Porites spp.* to be unaffected by OA (Edmunds & Yarid 2016).

Numerous previous studies focused solely on the effects of OA on species physiology and interactions (e.g. Glasl et al. 2016). However, IPCC reports highlight the joint effects of OA and warming that will affect future oceans. The goal of this study was to evaluate how the dual effects of these two factors affected coral-coral interactions. While it is not possible to make conclusions about the relative importance of the two environmental factors in this study, multiple studies have documented the immediate effect temperature stress can have on corals – particularly during the recent mass bleaching events (Hughes et

al. 2017). Follow-up studies considering the relative importance of these two factors and evaluating the generality of effects would be welcome.

Competitive interactions between *P. astreoides* and *M. cavernosa* or *O. faveolata* suggest a competitive hierarchy such as those found in earlier coral competitive interaction studies (Lang 1971, 1973). However, similar to the model evaluated by Horwitz et al.2017, these findings also suggest that competitive hierarchies may change or become more variable under OA and warming. If these changes occur in nature as oceans warm and acidify, this may establish a new competitive relationship among remaining corals on reefs in the Caribbean. *P. astreoides* is among the most abundant corals in the Florida Keys and is more abundant than either *O. faveolata* or *M. cavernosa* (Green et al. 2008, Burman et al. 2012). It was also relatively resistant to *O. faveolata* or *M. cavernosa* contact under present conditions; however, its advantage is lost as warming and acidification increase. *O. faveolata* is the least abundant of the three species (Burman et al. 2012), but it and *P. astreoides* had similar effects on each other's Fv/Fm (Fig 2.3), and *O. faveolata* caused larger lesions on *P. astreoides* than *P. astreoides* did on *O. faveolata* (Fig 2.5). These results suggest that the current dominant coral on Caribbean reefs may become more compromised under predicted future OA and warming.

Studies of coral-seaweed interactions also document competitive interactions being exacerbated by OA and warming (Anthony et al. 2011, Diaz-Pulido et al. 2011, Del Monaco et al. 2017). In our experiments, the effects of competition on *P. astreoides* were weaker under present day conditions than under OA and warming (Figs 2.1 and 2.3). The effects of OA and warming on coral-coral competition were species-specific, as also detected in other studies (Hughes et al. 2010, Del Monaco et al. 2017), making general

predictions of how OA and warming may affect these interactions difficult. In the context of these three species, the dominant aggressor under present conditions, *P. astreoides*, was disproportionately affected by the other two species under predicted future conditions.

Coral-coral interactions are less common on modern, degraded reefs; however, they still occur in localized patches where coral persists at higher cover (Horwitz et al. 2017). Understanding and predicting the outcome of these interactions may be of increasing relevance as rising temperatures and increasing OA alter community dynamics of future reefs.

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CHAPTER 3: EFFECTS OF OCEAN ACIDIFICATION AND WARMING ON A MORE TEMPERATE VERSUS MORE TROPICAL CORAL

Johnston NK, Burns AS, Hay ME. (2019) Effects of ocean acidification and warming on a more temperate versus more tropical coral. In Prep.

3.1 Abstract

Global climate change is predicted to transform ocean ecosystems as warmer temperatures and acidifying waters make these environments more inhospitable for many marine species. Some species may respond to these abiotic factors by shifting their ranges to newly hospitable environments; however, there have been few comparisons of how more temperate versus more tropical species may respond to shifting conditions resulting from climate change. Here, we evaluated how more temperate versus more tropical congeneric corals (*Oculina arbuscula* from temperate North Carolina and *Oculina diffusa* from subtropical Florida) responded to elevated temperature and carbon dioxide levels similar to those predicted to occur in coastal North Carolina within the next century. These congeneric corals from distinct environments and latitudes exhibited similar responses to OA and warming. Corals exposed to control conditions exhibited photosynthetic efficiencies 3-4x higher and growth rates 2-4.6x higher than corals exposed to warming and OA. The subtropical coral *O. diffusa* exhibited slightly greater reductions in photosynthetic efficiency due to future conditions than its temperate congeneric. These subtle differences in response to OA and warming may be linked to differences in

endosymbiont identity and density between the two congeners. Despite occurring in more stressful conditions of variable temperature, light, and turbidity, the temperate species was still susceptible to the effects of climate change.

3.2 Introduction

Climate change is stressing marine ecosystems (Hoegh-Guldberg and Bruno 2010, Doney et al. 2012). As oceans warm and acidify, species need to adapt, acclimate, or migrate in response to environmental stress, and scientists and resource managers are trying to predict the outcomes of such responses to better manage marine ecosystems and the services they provide (Hoegh-Guldberg et al. 2007, Hughes et al. 2017). Some species may survive climate change by shifting their ranges poleward to newly hospitable environments (Pecl et al. 2017). However, the introduction of novel species to these ecosystems comes with its own set of challenges and concerns including potentially dramatic changes in ecosystem composition and function (Marzloff et al. 2016). Range shifts are already occurring in some locations with dramatic consequences as the expansion of tropical herbivorous fishes to kelp forests in Australia shifted these ecosystems into seaweed barrens following the 2011 marine heatwave (Wernberg et al. 2016, Zarco-Perello et al. 2017) or to more tropical reef communities in Japan, Australia, and the Mediterranean over longer periods of warming (Vergés et al. 2014a, b, 2016). Such shifts are likely to become common across marine systems as species adapt to a changing climate via poleward migration (Pecl et al. 2017).

Similar to Pacific ecosystems, marine systems along the Atlantic coast of the United States are experiencing poleward shifts in fish assemblages (Nye et al. 2009, Vergés et al.

2014). Tropical species travel, often as larvae, along the Gulf Stream into temperate environments (Hare et al. 2002) but were traditionally unable to survive cooler winter temperatures (Hare & Able 2007). Warmer sea surface temperatures are changing these trends (Parker & Dixon 1998). Some tropical species, such as the grey snapper (*Lutjanus griseus*), are now commonly found in temperate habitats ranging from North Carolina to Cape Cod (Hare et al. 2012). The introduction of tropical species is likely to continue to increase as temperatures warm, but the consequences for these traditionally temperate ecosystems is unclear.

While most research of shifting habitat ranges under warming temperatures in marine systems has focused on fishes, climate change also may result in poleward shifts for other species. Coral larvae travel along coastal currents (Graham et al. 2008), and between 1974 and 2012 recruitment by corals increased by 78% in sub-tropical locations while decreasing by 85% in tropical areas (Price et al. 2019). Although potentially constrained by latitudinal attenuation of photosynthetically available radiation (Muir et al. 2015), it is possible that these range shifts will continue into temperate environments as subtropical habitats, such as the Florida Keys, become less hospitable due to warming (Manzello 2015). However, there is limited empirical evidence assessing this possibility or its potential consequences for more tropical versus more temperate species. Successful establishment of migrating species could also be impacted by increasing ocean acidification (OA) (van Hooidonk et al. 2014). OA can lead to coral bleaching, reduced growth, and death (Doney et al. 2009), and corals will be unable to escape this stressor simply by shifting to new environments. Comparative studies of how more tropical versus

more temperate corals may be affected by OA and warming along the southeastern coast of the United States are unavailable.

Coral bacterial microbiomes may play key roles in coral health via nutrient cycling and pathogen suppression (Ritchie 2006, Raina et al. 2016, Bourne et al. 2016, Beatty et al. 2019), and they could play a role in affecting how corals respond to OA and warming (Grottoli et al 2018). Similarly, endosymbiotic Symbiodiniaceae are important in coral stress response, and shifting identity or diversity of Symbiodiniaceae could be involved in coral acclimation to these stresses (Osman et al. 2020). For example, *Durusdinium spp.* (formerly clade D) is more thermotolerant and potentially protective against heat stress (Stat and Gates 2011) while *Symbiodinium spp.* (formerly clade A) is an opportunistic colonizer of heat-stressed corals that provides less nutrients and benefits to the host (Rouze et al. 2016). Thus, shifts in endosymbiont composition, density, or composition could occur as corals acclimate to OA and warming.

Oculina arbuscula is the dominant coral found on temperate reefs in the South Atlantic Bight, and *Oculina diffusa* is the only congener found at similar depths in the more tropical systems of the Florida Keys. Given that corals within this genus have facultative relationships with their *S*ymbiodiniaceae endosymbionts (Leal et al. 2014), focusing on these congeners allows for more direct comparisons of a warmer and colder water species without confounding taxonomy (i.e., different genera) or species having different relationships with their endosymbionts. Additionally, given the rarity of other coral genera in temperate regions of the North Atlantic Bight, the potential for light availability to limit further poleward migration of corals (Muir et al. 2015), and that most reefs in the North Atlantic bight are deeper and often poorly lit, a relative of *O. arbuscula* that is capable of

surviving on deeper, less well-lit reefs may be a more likely future colonizer. Our experiment sought to understand how congeneric corals collected from temperate (*O. arbuscula* - North Carolina) and subtropical (*O. diffusa* - Florida) environments survive the combination of elevated temperatures and ocean acidification likely to occur in the future, possibly providing insight into future interactions if the more tropical coral expanded poleward. We subjected both corals to future predicted levels of warming and OA and assessed their responses in terms of bleaching, growth, survival, and alterations of their bacterial microbiome and eukaryotic endosymbionts.

3.3 Methods

Corals were collected in December 2016 from shallow jetties off Morehead City, North Carolina (34-42'54" N , 076-41'06" W) and Summerland Key in the Florida Keys (24.6613° N, 81.4449° W) at depths of 2-5 m. Ten corals from each species were immediately preserved in RNAlater, put on ice, and stored in a -80 °C freezer when returned to the lab. These were used to document each of the coral's microbial and endosymbiont associates when initially collected from the wild. Approximately 50-60 coral fragments from each location were immediately placed in seawater-soaked bubble wrap, placed in a cooler to prevent thermal shock, and transported to a 20-tank system (37 L each) at the Georgia Institute of Technology in Atlanta, Georgia where tank temperature, pH, and lighting could be controlled. Coral fragments were cut into 4-6 cm fragments using a Dremel 4000 rotary tool, and fishing line was used to independently suspend each coral from a plastic grid attached to the top of each of the 20 tanks. Corals were hung using fishing line in a design similar to (Diaz-Pulido et al. 2011) rather than cemented to tiles on the bottom due to these corals' observed sensitivity to marine cements, which we observed

in preliminary assays. Five coral fragments of each species were randomly assigned to each tank to prevent confounding coral genotype with tank. Given that corals were collected during the colder, winter period, they were acclimated to tank environments while temperatures were slowly raised from 22°C to 28°C (present day summer temperatures off of North Carolina) over the course of six weeks.

After the six-week acclimation period, tanks were divided into two treatments – control (28.1°C, pH 8.11, & 609.76 pCO_2) and elevated (29.4°C, pH 7.81, & 1461.29 pCO_2) conditions (Table 3.1). Salinity differed between treatments, but only by 1.3 ppt (36.6 versus 37.9 in the control versus OA/warming treatment, respectively). This manipulation of temperature and pH was chosen to mimic a future coastal ocean predicted for the year ~2100 (Hoegh-Guldberg et al. 2014). Control tanks remained at their treatment levels throughout the experiment, while tanks with elevated temperature and OA had their temperature and OA levels increased slowly over two weeks (6 February - 20 February 2017). While the goal was to raise temperature and CO_2 levels slowly over two weeks to avoid shocking the corals, during the first day of acclimation, a break in the CO_2 line led to a spike across all tanks up to 1800 pCO_2 for a period of two hours as the room filled with CO_2 and affected control and elevated tanks. CO_2 levels were brought back down slowly (over a period of 24 h) using small water changes. The photosynthetic health of corals was monitored via a pulse-amplitude-modulated (PAM) fluorometer (see detailed explanation below). As this spike was of short duration, affected both elevated and present-day corals, and we observed no physical effects (bleaching, drops in PAM readings, etc.), we assumed that this event did not confound the experiment.

Table 3.1 Physical conditions for our two treatments. Shown are mean (±SE) temperature, salinity, and calculated carbonate parameters between ambient and elevated treatment conditions. P-values calculated using repeated measures ANOVA.

Climate	Temp (°C)	Salinity (ppt)	pH_{NBS}	pCO_2 (µatm)	CO_2 (µmol kg-1)	HCO_3 (µmol kg-1)	TA (µmol kg-1)	Ωca	Ωar
Ambient	28.14± 0.02	36.60± 0.25	8.11± 0.01	609.76± 16.61	15.83± 0.43	2192.37± 46.48	2801.98±5 9.05	6.08± 0.16	4.05± 0.12
Future	29.40± 0.06	37.87± 0.13	7.81± 0.05	1461.29± 43.70	36.51± 1.13	2690.16± 50.02	3105.55±5 1.64	4.15± 0.10	2.79± 0.13
p-value	p<0.001	p=0.001	p<0.001	p<0.001	p<0.001	p=0.002	p=0.080	p<0.001	p<0.001

Each 37-L tank in the OA system contained one 50-watt neo-therm heater to independently control temperature and one pump for continuous water circulation. Lighting was provided by banks of 4 tube lights; two were true actinic and two were T5 coral plus bulbs, providing a spectrum known to facilitate coral growth. They provided 210 µmol photons $m^{-2}s^{-1}$ to the bottom of the tank and cycled on a 12-hour light-dark cycle. A ThermoFisher Orion Star pH meter (relative accuracy ±0.01 units) was used to externally monitor and verify pH, temperature, and salinity daily. Saltwater was made using RO water and Instant Ocean Reef Crystals. Control tank CO_2 levels remained static in balance with the room. Elevated tank pH was continuously controlled and monitored using a pH stat computer (Aqua Medic), and pure carbon dioxide was bubbled into each tank (25 mL/min) as needed to maintain elevated treatment levels. Total alkalinity was measured weekly using an open-cell potentiometric titration from filtered (0.2 micron) water samples. Carbonate parameters within each tank were calculated using the CO2SYS program and the measured pH, total alkalinity, temperature and salinity parameters with the carbonate dissociation constants of Mehrbach et al. (1973) as refit by Dickson & Millero (1987).

Corals were fed hatched *Artemia* once a week on a day prior to a 25% water change, which occurred twice weekly with small additions of reverse-osmosis (RO) water added as needed to maintain salinity near 36 ppt.

Once all tanks were at appropriate temperature and carbon dioxide levels, an acclimated coral fragment of each coral species from each tank was collected (N=10 for each species and treatment), preserved in RNAlater, and frozen for later analysis to document the status of coral microbiomes and endosymbionts at the initiation of the experiment. By comparison to the samples taken immediately in the field, this allowed an assessment of changes in microbiome composition due to tank acclimation prior to our experiment. The effects of increased temperature and OA on coral health, growth, and survival were evaluated over the course of a seven-week experiment (21 February 2017-6 April 2017). Coral photosynthetic health was assessed weekly using PAM fluorometry to evaluate endosymbiont photosynthetic potential (Fv/Fm). Corals were dark-adapted for two hours before Fv/Fm was measured for each coral. Three PAM readings were taken randomly over each coral, with the average of those readings taken as the Fv/Fm for that coral fragment. Photosynthetic efficiency was evaluated across the entire population (i.e., considering dead corals as having a zero PAM reading), but also for only the remaining survivors (i.e., excluding dead corals from PAM assessments).

Coral growth was determined every three weeks via mass measurements. Skeletal growth was determined via the buoyant-weight method (Davies 1989). Corals also were lightly shaken twenty times and the coral's total weight mass recorded (assessing both skeletal and wet tissue mass; Clements and Hay 2019). Tissue mass was determined by subtracting the coral's buoyant weight from its total wet weight. At times, filamentous

algae started colonizing dead areas of a coral. When this happened, the filamentous algae were removed with gentle brushing before weighing. Changes in coral growth were evaluated at the population level (where the mass of the dead corals were included) and for surviving individual alone (where dead individuals were excluded from the analysis).

Evidence for death was monitored daily. Corals in the genus *Oculina* can survive without symbionts making loss of color inadequate for evaluating tissue mortality. Corals were considered dead only once they fulfilled three criteria: the entire coral fragment lacked coloration, all coral tentacles failed to respond to the presence of food (i.e. tentacles did not emerge when squirted with seawater from *Artemia* spp. cultures), and the coral failed to produce mucus. Dead corals were immediately removed from the tanks to avoid degradation of water quality.

At the end of the seven-week experiment, all surviving corals were preserved in RNAlater and stored at -80°C for endosymbiont and microbiome analysis. Tissues from all time points (wild, post-acclimation, and at the experiment's end) were removed individually from thawed corals using a WaterPik (Johannes and Wiebe 1970), and the tissue slurry was centrifuged at 10,000 RPM for 10 minutes. The resulting tissue pellet was weighed, homogenized in 5 mL of sterile seawater using a tissue homogenizer, and vortexed. Three mL of tissue and seawater was preserved in 0.05% glutaraldehyde for endosymbiont density analysis while the remaining 2.0 mL was used for microbiome analysis (see below). In some cases, the mucus formed a dense foam at the top of the tube following centrifugation. Due to the high-water content, this dense foam was not included in the weight, but it was included in the homogenate. Endosymbiont density was then

determined using a hemocytometer, and the average number of cells per gram of removed tissue was calculated.

Data analyses were completed using the R statistical software version 3.6.0 using the car 2.1-5 (Fox & Weisberg 2019), lme4 1.1-21 (Bates et al. 2015), emmeans 1.4 (Lenth 2019). and coxme 2.2-1.4 statistical packages (Therneau 2020). Between treatment differences in temperature, pH, carbonate chemistry parameters, temperature, and salinity were evaluated using repeated measures ANOVA for each variable. The effects of OA/warming and species identity on endosymbiont Fv/Fm values over time, end-point endosymbiont density, and coral growth (buoyant weight, tissue growth, and total growth) were analyzed using linear mixed models to include tank as a random and blocking factor. Post-hoc tests were conducted on Fv/Fm values over time using mixed models on individual comparisons. P-values were corrected using the Bonferonni correction. Differences in endosymbiont density from time of collection to experiment initiation were evaluated using generalized linear models. The effect of OA/warming on final endosymbiont density was evaluated separately using linear mixed models. Coral survival across treatments was evaluated via a proportional hazard model using the coxme R statistical package to include tank as a random factor.

Microbiome analysis

Two ml of tissue homogenate from each sample was transferred to a microcentrifuge tube and centrifuged at 13,000 rpm for five min. The resultant pellets were added to PowerBead tubes from a PowerSoil DNA Isolation Kit (Qiagen) and DNA was

extracted according to the manufacturer's instructions. Purified DNA was quantified using a Qubit 2.0 fluorometer (ThermoFisher Scientific).

For the bacterial component of the microbiome, the V3-V4 hypervariable region of the 16S rRNA gene was amplified using universal 16S rRNA gene primers 515F (Parada) (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (Apprill) (5'-GGACTACNVGGGTWTCTAAT-3'). For the *Symbiodiniaceae* component, the internal transcribed spacer 2 (ITS2) region of the nuclear ribosomal RNA was amplified using primers itsD (5'-GTGAATTGCAGAACTCCGTG-3') and its2rev2 (5'-CCTCCGCTTACTTATATGCTT-3') (Cunning 2017, Stat 2009). Primers were modified with sample-specific barcode sequences and Illumina-sequencing adapters according to Kozich et al. (2013).

For each sample, 1.5 µl of DNA (total reaction volume of 25 µl) was amplified using GoTaq DNA Polymerase (ThermoFisher Scientific) with a final primer concentration of 0.2 µM and 10 µg of bovine serum albumin (BSA; New England Biolabs, Inc.) added as a PCR enhancer. For bacterial samples, PCR cycling conditions were an initial denaturation at 95°C for 3 min, followed by 30 cycles of a 45-s denaturation step (95°C), 45-s primer annealing step (55°C), and 90-s extension step (72°C), with a final extension step of 10 min at 72°C. For *Symbiodiniaceae* samples, PCR cycling conditions were an initial denaturation at 95°C for 3 min, followed by 30 cycles of a 45-s denaturation step (95°C), 45-s primer annealing step (57°C), and 90-s extension step (72°C), with a final extension step of 10 min at 72°C. PCR products were run on a 1% agarose-Tris-acetate-EDTA (TAE) gel along with no DNA negative controls to verify amplicon size and the absence of contamination. The products were purified using the QIAquick PCR

Purification kit (Qiagen, Hilden, Germany) and quantified using the Qubit 2.0 fluorometer. Equimolar concentrations of each amplicon were pooled, mixed with 10% PhiX DNA to increase template diversity and sequenced using a 500-cycle paired-end MiSeq reagent V2 kit on an Illumina sequencer.

After demultiplexing, barcoded sequences were trimmed and filtered using Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/, Phred score > 25, minimum sequence length >100 nt). Paired-end sequences were merged and exact sequence variants (ESVs) were determined from filtered sequences using DADA2 in the QIIME2 pipeline (Callahan 2016, Bolyen et al., 2019). For bacterial sequences, taxonomy was assigned to ESVs by comparison to the SILVA ribosomal RNA database (Release 132). For *Symbiodiniaceae* sequences, taxonomy was assigned by comparison to a custom-built database (see below for full parameters of the database). Singletons and ESVs assigned to chloroplast, mitochondria, or coral sequences were removed from further analyses. Alpha- and beta-diversity metrics were calculated in the R package vegan (v2.5-6) and plotted in the R package phyloseq (v.1.28.0) (McMurdie 2013). Differential abundance of ESVs and higher taxonomic levels was calculated in the R package DESeq2 (v.1.24.0) (Love and Huber, 2014).

The custom database was built by first querying the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) using the keywords “symbiodinium[Organism]" and "ITS2" (Nov 2018). Sequences were retained if they contained both primers with a sequence identity >75% and then all regions of the sequences outside the primers were trimmed using the script cutprimers.sh from the bbtools package

(https://sourceforge.net/projects/bbmap/). The resultant fasta file was used to train a Naive Bayesian classifier using QIIME2.

3.4 Results

The coral species responded differently to the two climate conditions. Photosynthetic efficiency, measured as Fv/Fm, showed similar trends whether we considered all coral through time (Fig 3.1A) or excluded corals that died as the experiment progressed (Fig 3.1B). Climate did not interact significantly with species over time (Climate*Species*Time: $p=0.940$, Figure 3.1), but the climate*species interaction was significant when considering both all corals and only surviving corals ($p=0.006$ and $p=0.003$, respectively). Post-hoc analysis indicated that Fv/Fm values for *Oculina arbuscula* (North Carolina) remained higher than for *Oculina diffusa* (Florida) under elevated conditions when considering all corals ($p<0.001$), but these differences lost significance when considering only those corals that survived the entire experiment ($p=0.345$). *O. arbuscula* (North Carolina) and *O. diffusa* (Florida) both exhibited significantly lower Fv/Fm values when exposed to elevated OA/warming compared to control conditions starting at about weeks five to six and continuing through the end of the experiment (Figure 3.1A&B, Climate*Time: $p<0.001$). By the end of the experiment, corals exposed to ambient conditions exhibited photosynthetic efficiencies 3.2 (*O. arbuscula* – North Carolina) to 4.2 times (*O. diffusa* - Florida) higher than conspecifics experiencing elevated OA and warming conditions.

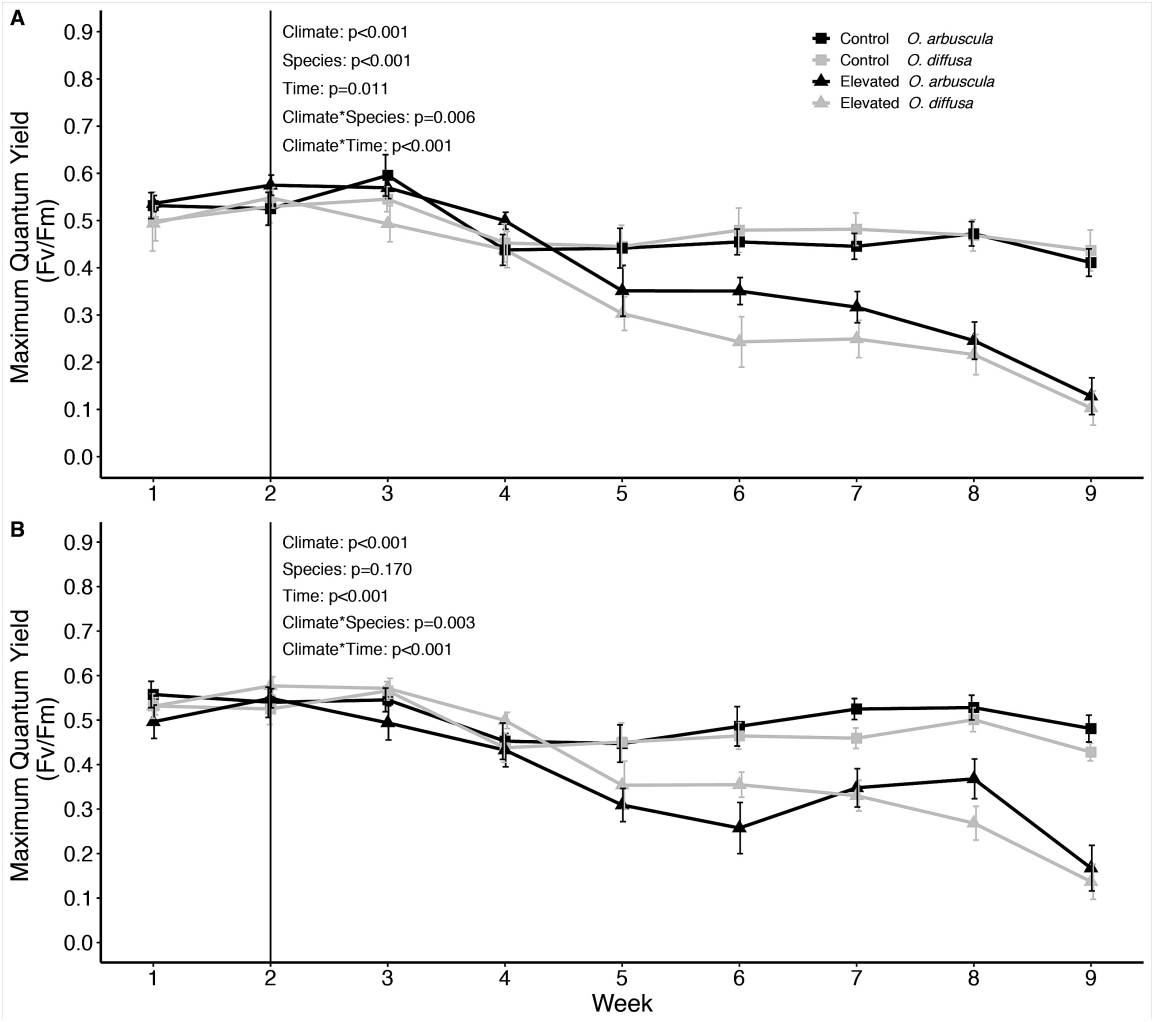

Figure 3.1 The effect of elevated carbon dioxide and temperature on endosymbiont photosynthetic efficiency for *O. arbuscula* (NC) and *O. diffusa* (FL) when all corals are included with dead corals scoring zero (A) and excluding dead corals (B). Shown are means (±SE). The vertical line at week 2 separates the acclimation and experiment conditions.

Coral survival did not differ significantly as a function of either species or treatment, although there was a nearly significant effect of treatment (Figure 3.2, Climate: p=0.065). By the end of the experiment, mortality of corals exposed to elevated temperature and OA were 11% (NC) to 35% (FL) greater than conspecifics exposed to control conditions, but these trends were not statistically significant.

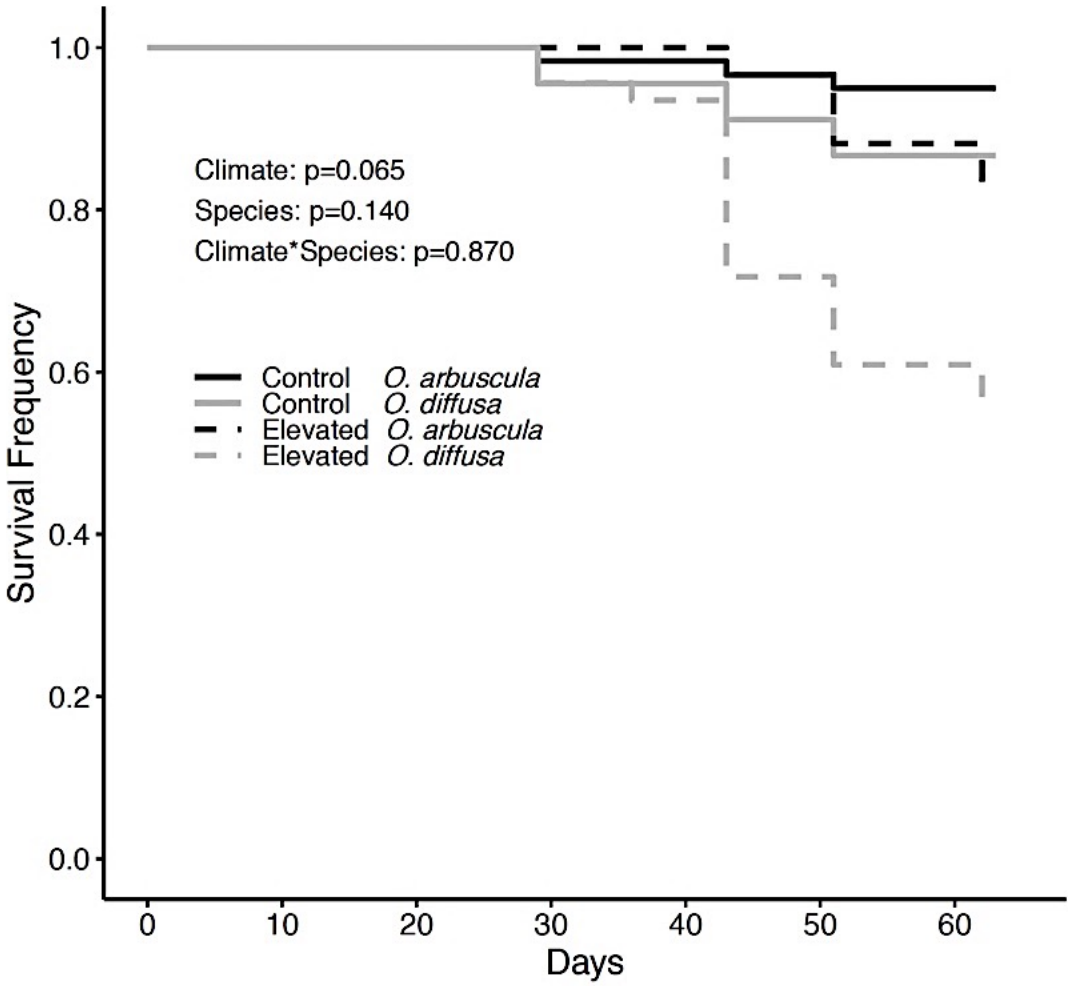

Figure 3.2 The effect of elevated carbon dioxide and temperature on coral survival across the two coral species.

Considering both all corals and only those that lived, *O. diffusa* (Florida) calcified at a rate of 1.5-4.3 times greater than *O. arbuscula* (North Carolina; p=0.05 and 0.48, respectively, Figure 3.3A&B). Elevated temperature and OA did not significantly affect buoyant weight (Figure 3.3A&B). Tissue growth was unaffected by OA and warming for both species (Figure 3.3C&D). OA and warming did significantly suppress population total growth in mass with ambient corals' growth rates 2.1-4.6 times higher than corals exposed to elevated OA/warming when considering all corals (Figure 3.3E: p=0.005), but not when considering only those alive at the end of the experiment (Figure 3.3F; p=0.267).

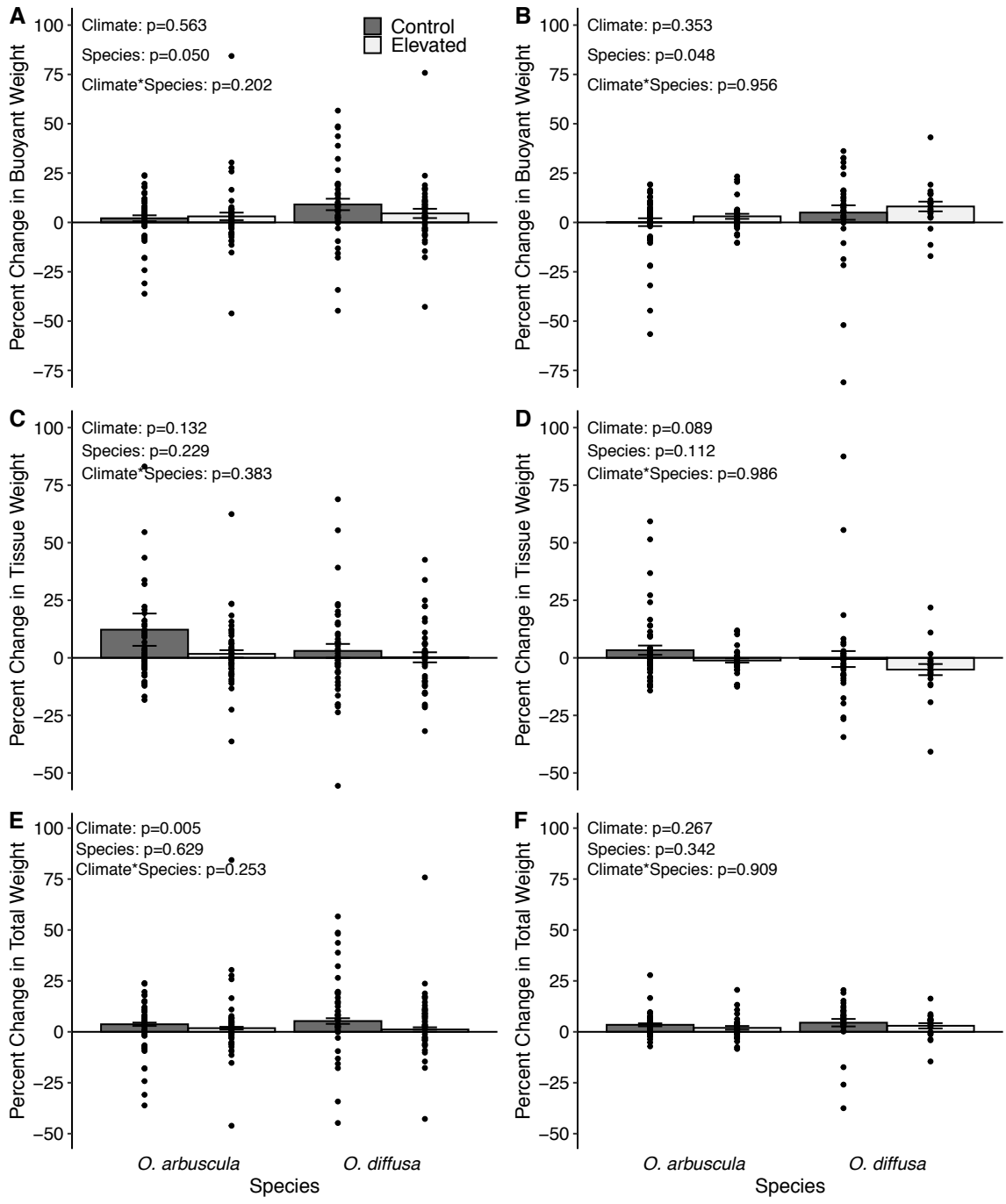

Figure 3. 3 The effect of OA and warming on coral buoyant weight (skeletal growth) (A,B), tissue growth (C,D), and total growth (E,F) for the two species when all corals are included (alive and dead) (A,C,E) and when only living corals are included (B,D,F). Shown are means (±SE). Dots represent individual data points.

Endosymbiont density at the time of collection was 3.5x greater in *O. diffusa* (Florida) versus *O. arbuscula* (North Carolina) ($1.29x10^7$ versus $3.72x10^6$ cells/g, respectively; $p<0.001$; Figure 3.4A). After 2 months of acclimation in the lab, values had dropped 50 to 58% (for the Florida and North Carolina species, respectively) and were $5.39x10^6$ cells/g (*O. diffusa)* and $1.87x10^6$ cells/g (*O. arbuscula)* at the start of the experimental manipulation. The species continued to differ significantly in endosymbiotic density at the end of the experiment ($p<0.001$); effects of elevated temperature and OA were not significant but there was a trend for lower endosymbiont density in the elevated conditions ($p=0.066$) (Figure 3.4B). Endosymbiont density was 46-64% lower in *O. arbuscula* versus *O. diffusa* ($p<0.001$) at the end of the experiment.

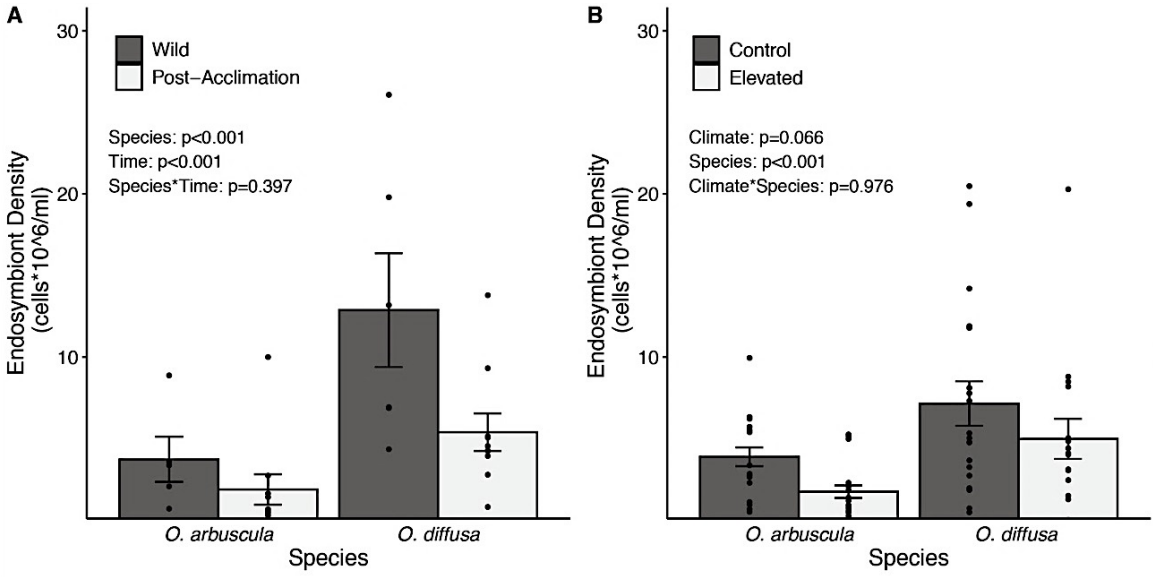

Figure 3.4 The effects of elevated carbon dioxide and temperature on endosymbiont density (mean±SE) for the two coral species. A) Wild and Post-Acclimation B) End of experiment. Dots represent individual data points.

Corals differed significantly in Symbiodiniaceae species richness and composition as a function of treatment, with controls having higher exact sequence variants (ESV) richness than elevated treatments ($p=0.025$); but no significant difference occurred in their Shannon index ($p=0.528$) (Figure B1). When comparing the endosymbiont community of *O. arbuscula* (North Carolina) to *O. diffusa* (Florida) there were significant differences in both ESV richness ($p=0.045$) and Shannon index ($p=0.002$) (Figure B2). Symbiodiniaceae community composition did not differ as a function of treatment (ANOSIM, R: 0.02076, $p=0.133$) but did differ between coral species (ANOSIM, R: 0.4921, $p<.001$; Figure 3.5A). The between-species difference in Symbiodiniaceae community structure was driven primarily by two ESVs. Symbiodiniaceae ESV1 comprised 73.1% (elevated treatment) and 79.2% (control treatment) of all sequences from *O. arbuscula* (North Carolina), while ESV2 comprised 70.8% (elevated treatment) and 67.1% (control treatment) of the dinoflagellates in *O. diffusa* (Florida) (Figure 3.5B). For the majority of individual coral samples, a dominant Symbiodiniaceae ESV made up >80% of the total community with the average declining for unusual individuals showing dominance of the "other" corals' ESV (i.e. an *O. diffusa* coral with a dominant *O. arbuscula* symbiont).

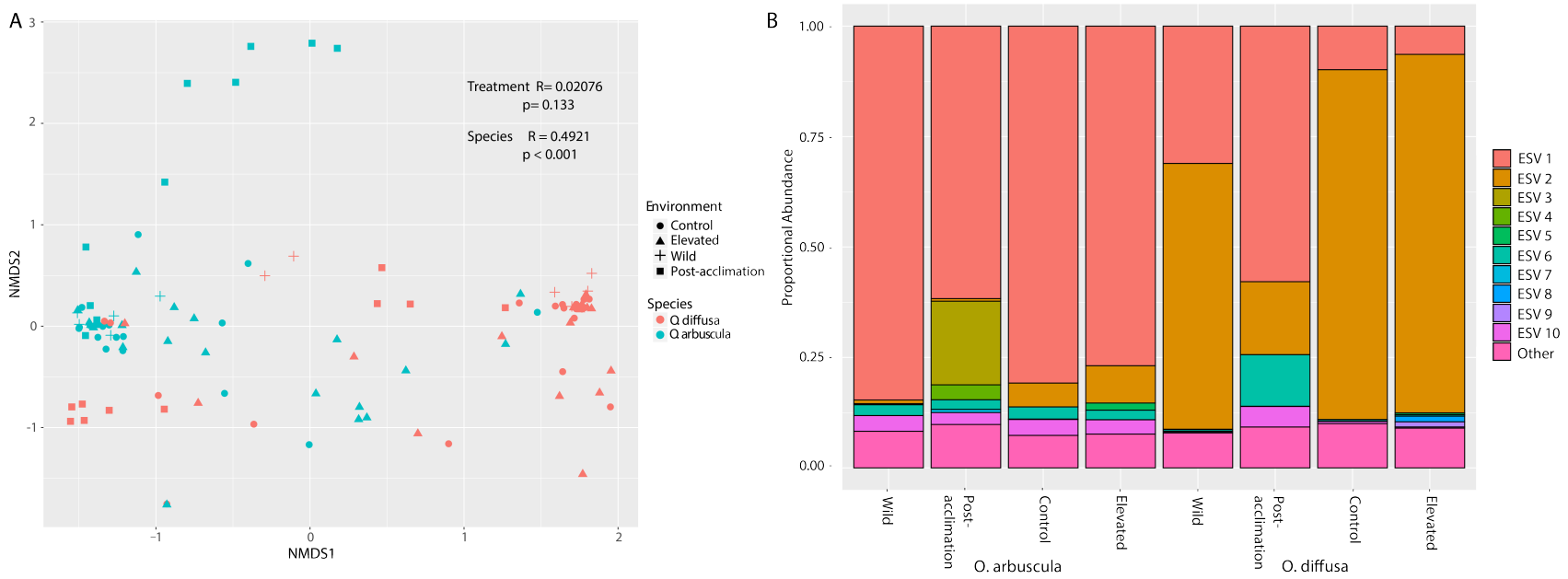

Figure 3.5: (A) NMDS plot of endosymbiont composition (Bray-Curtis dissimilarity). (B) Proportional representation of the top ten most abundant endosymbiont averaged by sample type. All other exact sequence variants (ESVs) grouped into the Other category.

Based on phylogenetic analysis of the ITS2 region, both ESV1 and ESV2 are members of the genus *Breviolum* within the Symbiodiniacea*e* family with ESV2 most closely related to *Breviolum minutum* and ESV1 most closely related to *Breviolum psygmophilum,* two species forming distinct linearges within the *Breviolum* physlogenetic tree (Lajeunesse et al. 2018). Only four other Symbiodiniaceae ESVs showed enrichment in either coral species. ESV25 (7 $\log_2$ fold-change, FDR-adjusted p-value: <.001) and ESV14 (3.4 $\log_2$ fold-change, FDR-adjusted p-value: <.001) were closely related to ESV2 and were more abundant in *O. diffusa* (Florida) corals. Two ESVs closely related to ESV1, ESV15 (3.0 $\log_2$ fold-change, FDR-adjusted p-value: <0.001) and ESV18 (2.7 $\log_2$ fold-change, FDR-adjusted p-value: <0.001), were similarly enriched in *O. arbuscula* (North Carolina) corals compared to their Florida counterparts.

Values of ESV1 and Fv/Fm were positively correlated (R= 0.34 p=0.005) (Figure 3.6A), but no significant relationship occurred Fv/Fm and ESV2 (R= 0.091, p=0.46) (Figure 3.6B).

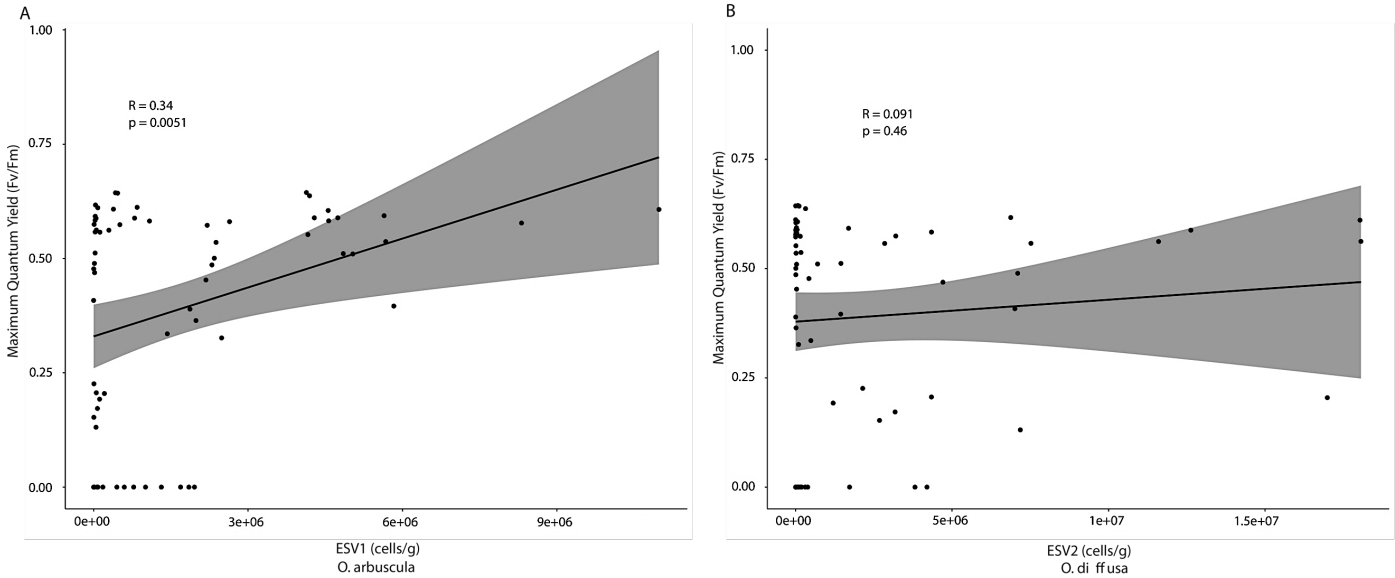

Figure 3.6 Correlation of dominant emdosymbiont ESVs with maximum quantum yield. (A) ESV1 (*O. arbuscula)* (B) ESV2 (*O. diffusa).* Lines represent linear regression with shaded regions representing 95% confidence intevals

Coral microbiomes did not differ significantly in species composition due to either coral species (ANOSIM, R: -0.01876, p=0.939) or treatment (ANOSIM, R: 0.001593, p=0.357) across sampling periods (wild, post-acclimation, experiment conclusion Figure 3.7A). There were also no detectable differences in microbiome diversity as a function of treatment (ESVs richness, p=0.393, Shannon diversity, p=0.441, Figure B3) or coral species (ESVs richness, p=0.238, Shannon diversity, p=0.293, Figure B4). Composition variability also did not change with treatment or species (PERMDISP2, treatment: p=0.367 species: p=0.910, Figures B5-B6). Comparison of bacterial samples at the family level indicated similar microbial composition across all samples with Alteromonadaceae, Rhodobacteraceae, and the Oceanospiralles family SS1-B-06-26 as the most abundant taxa (Figure 3.7B). Differential abundance of taxonomic groups at the family, genus, and ESV level using DESeq2 indicated no taxa enriched between treatment groups or treatment-coral species pairs. DESeq2 did indicate an enrichment within *O. arbuscula* (North Carolina) corals of an uncultured bacterium within the Oceanospiralles family SS1-B-06-26 (23.2 log2 fold change, FDR-adjusted p<.001). This enrichment was driven primarily by a single ESV that was 22.03 log2 fold higher (FDR-adjusted p<.001) in *O. arbuscula* (North Carolina) microbiomes compared to *O. diffusa* (Florida).

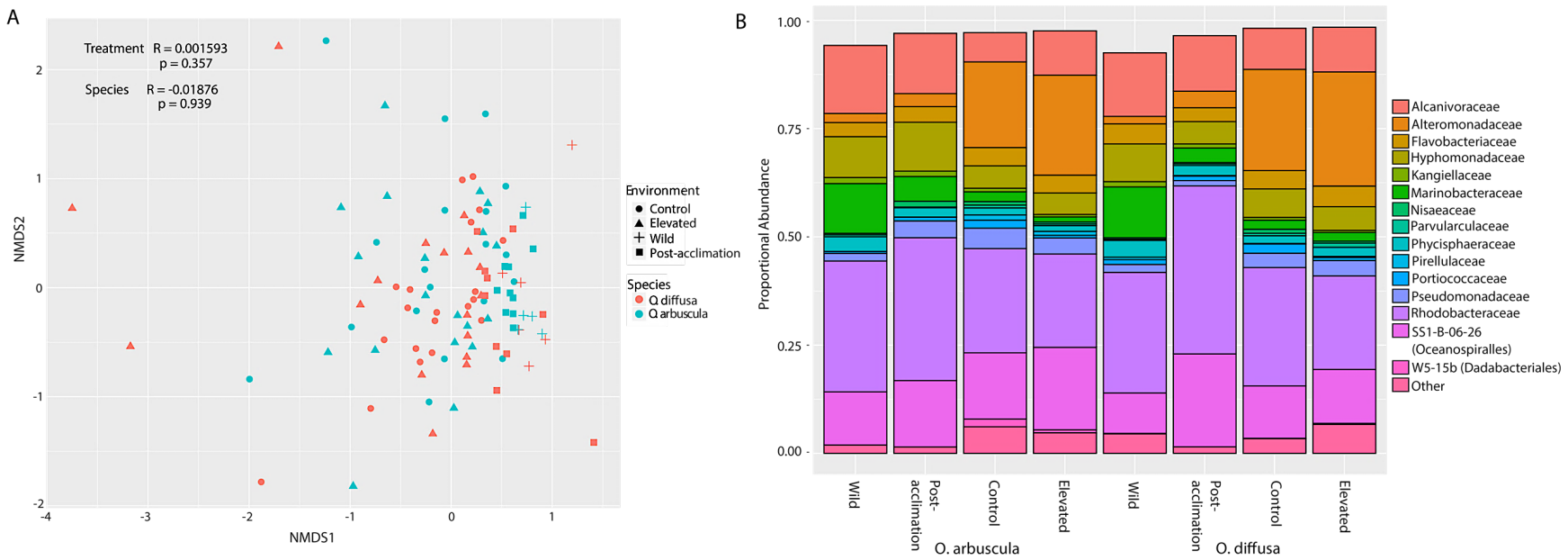

Figure 3.7(A) NMDS plot of bacterial microbiome composition (Bray-Curtis dissimilarity). (B) Proportional representation of the top ten most abundant bacterial families averaved by sample type. All other families grouped in the Other category.

3.5 Discussion

Both *Oculina arbuscula* (NC) and *Oculina diffusa* (FL) experienced reduced endosymbiont density (Figure 3.4), reduced photosynthetic efficiency (Figure 3.1), and reduced total growth (Figure 3.3) when exposed to elevated OA and warming. The tropical *O. diffusa* was slightly, but significantly, more impacted by OA and warming in terms of Fv/Fm (Figure 3.1) and possibly survivorship (Figure 3.2) than its temperate congener, but total growth did not differ between the two and skeletal growth (buoyant weight) remained higher for the more tropical species (Figure 3.3). Overall, neither the temperate nor more tropical coral were resistant to the negative effects of climate change.

The CO_2 levels in this study were higher than predicted values for 2050 (current levels=408 pCO_2 vs ~607 pCO_2 in our control tanks and worst-case-scenario at the year 2100 of ~1250pCO_2 – IPCC RCP 8.5 vs our experimental of ~1400 pCO_2) due to the higher alkalinity of the Instant Ocean Reef Crystals used to make seawater. However, both species were collected from nearshore environments that may have more variable abiotic conditions that could lead to naturally higher CO_2 levels than the predicted values which are based on open ocean parameters (Frieder et al. 2012, Duarte et al. 2013, Reum et al. 2014). Control corals did not exhibit significant reductions in PAM readings by the end of the experiment compared to their initial values suggesting that 607 pCO_2 was not causing coral bleaching (unlike the decreases that occurred under elevated conditions – Figure 3.1). Despite both corals responding poorly to increased OA/warming, *O. arbuscula* (North Carolina) exhibited significantly higher Fv/Fm values under elevated conditions than did *O. diffusa* (Florida) over the course of the experiment (Figure 3.1A&B). However, given the modest magnitude of the differences measured and post-hoc analysis that failed to find

a significant difference in Fv/Fm values between the two coral species, O. *arbuscula* (North Carolina) appears only marginally more resistant to climate change in terms of effects on Fv/Fm than *O. diffusa* (Florida).

Skeletal growth was unaffected by OA/warming, however the two coral species exhibited significantly different buoyant weights, with the more tropical species calcifying more rapidly; this relationship occurred both for all corals and when considering only surviving corals. Reasons for this difference were not investigated, but differences in energy acquisition between the two corals may be a factor. Corals primarily utilize resources from their endosymbionts for skeletal growth, and differences in endosymbionts' identity, chlorophyll *a* concentration, or photosynthetic efficiency may contribute to differences in buoyant weight (Hughes et al. 2010, Iguchi et al. 2012).

The endosymbiont density of *O. diffusa* (Florida) was 1.9 times (end of experiment control *O. arbuscula* vs end of experiment control *O. diffusa* – Figure 3.4B) to 3.5 times higher (Wild *O. arbuscula* vs Wild *O. diffusa* – Figure 3.4A) than its congener from NC (Figure 3.4). While endosymbiont density decreased between the time of collection and of experiment initiation, it is likely a consequence of increasing temperatures to summer levels (Aichelman et al. 2016), or other parameters such as altered light levels associated with laboratory conditions. At the end of the experiment, both corals still harbored significantly different endosymbiont densities despite having similar Fv/Fm values at each time point (Figure 3.1). These results suggest that *O. arbuscula* (North Carolina) possesses endosymbionts with superior photosynthetic efficiencies (Iguchi et al. 2012) relative to those from *O. diffusa* (Florida), possibly due to local adaptation to a more variable environment in terms of temperature, turbidity, and probably other factors (e.g., salinity).

In some cases, corals from more stressful areas have exhibited greater resistance to bleaching (Morgan et al 2017, Camp et al. 2018), and higher endosymbiont density has been linked with increased susceptibility to bleaching (Cunning & Baker 2013). *O. arbuscula*'s (North Carolina) lower endosymbiont density relative to *O. diffusa* (Florida) may thus also be evidence of a marginally more stress resistant coral.

As with other studies focusing on endosymbionts in corals in the genus *Oculina,* the corals in our experiment were dominated by Symbiodiniaceae in the genus *Breviolum* (previously clade B) (LaJeunesse 2001, Lajeunesse et al. 2012, Leydet and Hellberg 2016, LaJeunesse et al. 2018) – considered to be one of the less thermotolerant clades (Silverstein et al. 2012). Our findings for *O. arbuscula* (North Carolina) supported other research documenting that many *Oculina* are dominated by ITS Type B2 - specifically *Breviolum psygmophilum* (Leydet and Hellberg 2016). However, as with Leydet & Hellberg (2016) who found unique populations of *Oculina* to harbor ITS2 type B1 (*B. minutum* specifically), *O. diffusa* contained *B. minutum*. Moreover, our results found that in the wild and at the end of each experiment each *Oculina* species contained their respective ESVs of these species at greater than 70% relative abundance (Figure 3.5B). However, post-acclimation corals from both species exhibited significant reductions in the dominant ESV. It is possible that collection and acclimation resulted in a suppression of the dominant ESV that recovered over the course of the experiment to a steady state. These results suggest that *O. arbuscula* and *O. diffusa* may be unable to acclimatize to environmental stress by symbiont switching as has been suggested in other species (Jones et al. 2008).

The marginally different responses in Fv/Fm that *O. diffusa* (Florida) and *O. arbuscula* exhibited in response to OA/temperature elevation (Figure 3.1) may also result

from a difference in their endosymbiont identity rather than density. The density of the major endosymbiont clade (EVS1) in the North Carolina coral correlated with photosynthetic efficiency ($p=0.005$; $R=0.34$), while no such correlation occurred for the major endosymbiont (ESV2) in the Florida coral ($p=0.46$; $R= 0.09$; Figure 3.6). These divergent relationships indicate that these two ESVs differ in their photosynthetic efficiency, explaining how *O. arbuscula* (North Carolina) and *O. diffusa* (Florida) exhibit equivalent Fv/Fm levels at the end of the experiment despite *O. diffusa* having higher endosymbiont densities. The relative abundances and identity of the dominant endosymbiont for each *Oculina* were not correlated with resistance to OA/warming suggesting that neither of these two ESVs or others found at lower relative abundance (Silverstein et al. 2012) confer strong resistance to OA/warming as has been suggested with some other clades (Fabricius et al. 2004). Overall, our results suggest that the relationship between endosymbiont identity, density, and bleaching propensity is complicated, and resistance to bleaching may not be tied to any one factor.

Coral microbiomes also may influence a coral's bleaching response to environmental stressors (Fabricius et al 2004, Abrego et al. 2008, Oliver & Palumbi 2011, Smith et al. 2017). The bacterial microbiome was not significantly influenced by OA/temperature (Figure 3.7A), and only one ESV, closely related to an uncultured Oceanospiralles, was enriched in *O. arbuscula* (North Carolina) samples compared to *O. diffusa* (Florida). These results mirror some research that found that microbial community structure can remain stable under different OA conditions (Zhou et al. 2016) but stand in contrast to other studies that found significant shifts in the microbial community in response to natural coral bleaching (Vega Thurber et al. 2009, Littman et al. 2011). The

explanation for the differences between this study and others is unclear. However, some have cautioned against drawing too close of a link between a coral microbiome and its susceptibility to bleaching (Mouchka et al. 2010), and changes in coral microbiomes post-bleaching may be a response to, rather than a cause of, bleaching.

It is not possible to generalize about the ability of subtropical versus tropical corals to survive climate change by shifting their habitats given that only one species from each habitat was studied in this experiment. However, studies in other organisms have shown that more tropical species are expanding into more temperate environments as climate rigors change (Nye et al. 2009, Vergés et al. 2014, Marzloff et al. 2016). Moreover, it should be noted that these two species were chosen for contrast because this Caribbean species was a related congener and like its North Carolina relative was relatively tolerant of low light, and thus more likely to be suited to the more turbid waters further north; both corals occur in stressful turbid environments (Leydet and Hellberg 2016) and have facultative relationship with their endosymbiont (Leal et al. 2014).

Unlike other studies considering the ability of tropical species to migrate to temperate habitats that focused mainly on temperature (Nye et al. 2009, Wuenschel et al. 2012), our experiment evaluated the joint effect of predicted increases in temperature and OA on coral physiology to better understand how congeneric corals from more tropical versus more temperate areas might respond to future conditions. While this temperature stress was not outside of the realm of average summer water temperatures in the Florida Keys, it was 1.5°C above North Carolina summer water temperatures (NOAA National Data Buoy Center – Station BFTN7). However, we detected no evidence of the more tropical coral being advantaged over the more temperate species under increased

temperature and OA. In some species, OA and warming act synergistically to increase coral mortality (Prada et al. 2017) and negatively impact coral physiology (Schoepf et al. 2013, Grottoli et al. 2018). For our test species, warming and OA generally impacted both species in a similar manner, decreasing both photosynthetic efficiency and total growth.

The relationship between corals, their endosymbionts, and their microbial associates is complicated, and neither endosymbionts nor microbiomes may be the best indicator of a coral's susceptibility to changing oceans. In this contrast, the temperate coral, *O. arbuscula*, that is well-adapted to stressful and variable environments appeared no more resistant to climate induced stresses than its more tropical relative.

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CHAPTER 4: RESPONSE OF A TEMPERATE CORAL TO TEMPERATURE STRESS AS A FUNCTION OF DEPTH OF COLLECTION

Johnston NK, Burns, AS, Hay ME (2019) Depth and temperate coral resistance to ocean warming. In prep.

4.1 Abstract

Climate change is a threat facing many coral reefs. Coral susceptibility to climate change is dependent on many physiological and environmental factors, and shallow, tropical corals are often thought to be the most susceptible due to their location at the upper limit of their thermotolerance. Environments where corals live but do not dominate, such as temperate or deep-water environments, are often thought to be more resistant to thermal stress. These environments may be natural refuges from elevated temperatures or experience greater environmental variation that could enhance coral resistance to thermal stress. Yet, recent studies demonstrate mixed responses to climate change for corals from temperate and/or deeper water environments. For temperate reefs, coral response to thermal stress may depend on small-scale, local environmental factors, such as depth, that impact resistance. We studied whether physiological response of the temperate coral *Oculina arbuscula* to thermal stress (+1.4°C above average summer water temperatures) depended on the depth from which the corals were collected (less than nine meters or greater than 20 meters deep). Both corals exhibited reduced photosynthetic efficiency and endosymbiont density under thermal stress and lab conditions. However, deep-water *O. arbuscula* exhibited greater reductions in tissue growth and endosymbiont density than its shallow

water conspecifics. Environmental variation in shallow locations may enhance the resistance of shallow water corals to thermal stress relative to deep-water corals.

4.2 Introduction

Global climate change will cause an increase in the frequency and duration of marine heatwaves and an overall increase in average sea surface temperature (Frölicher et al. 2018). For tropical corals, which serve as foundation species and commonly exist near the upper limits of their thermotolerance (Fitt et al. 2008), extended temperature stress can lead to reduced growth, bleaching, and mortality (Hughes et al. 2017). Corals vary in their susceptibility to temperature stress (Guest et al. 2012), and certain physiological (e.g. phenotypic plasticity, microbiome) and environmental factors (e.g. temperature, turbidity, and fluctuations in these factors in shallow, near-shore environments) are correlated with, and may enhance, a coral's ability to withstand extended high temperatures (reviewed in Camp et al. 2018). As tropical reefs become less hospitable to coral survival, traditionally less favorable locations, such as temperate or deeper reefs, may see enhanced coral growth and, possibly, the arrival of non-native species spreading poleward (Bongaert et al. 2019, Verges et al. 2014). It is not clear how more temperate corals already found in these locations may respond to a changing climate.

Although less well-studied than tropical corals, temperate corals are now being evaluated for their susceptibility to climate change (Wernberg et al. 2011). It has been suggested that corals found in temperate reef environments may be less susceptible to temperature stress than their tropical counterparts due to a history of exposure to greater

environmental variation, which may enhance climate acclimation or adaptation to variable temperatures (Beger et al. 2014). However, studies on temperate corals demonstrate mixed responses to increased temperatures (Rodolfo-Metalpa et al. 2005,2008, Kersting et al. 2015, Aichelman et al. 2016), and it is unclear how increased temperature will impact temperate coral survival and growth.

It is possible that variances among different temperate reef habitats may influence coral susceptibility to temperature stress. Experiments on tropical reefs found that corals from turbid, near-shore waters and shallow, high temperature pools can withstand greater temperature stress than conspecifics from more stable environments (Palumbi et al. 2014, Morgan et al. 2016). Temperate marine environments can vary in temperature, turbidity, light availability, and nutrient input, etc. within the span of a few kilometers (Atkinson 1983,Garrabou et al 2002), and conspecifics from different, but nearby, locations can be exposed to vastly different environmental conditions that affect their physiology (Garrabou et al. 2002, Özalp et al. 2018) In some places, such as the temperate reefs of the South Atlantic Bight, these varying environmental conditions are tied to depth (Atkinson 1983). Some studies suggest that deeper reefs may serve as refuges for temperature-stressed shallow corals (Bongaert & Smith 2018). Corals already found on deeper reefs may bleach less often since they are exposed to temperature stress less frequently than shallow corals (Penin et al. 2007, Smith et al. 2014, Muir et al. 2017), but they are also at greater risk of bleaching than shallow corals when exposed to temperature stress (Torrents et al. 2008, Pey et al. 2011, Smith et al. 2015). While these studies are suggestive of depth-dependent impacts of temperature stress on corals, results are mixed and limited to a few geographic locations.

Oculina arbuscula is the most common warm-temperate coral on natural and artificial reefs in the South Atlantic Bight; it is facultative with its photosynthetic endosymbiont and occurs at depths of less than 5 m near shore to greater than 30 m on off-shore reefs and ledges (Leal et al. 2014). Near-shore, shallow locations are characterized by more variable salinity, larger temperature differences, greater turbidity, and enhanced nutrients compared to deeper locations (Atkinson et al. 1983, Blanton et al. 2003), and corals in these shallow locations might be expected to be more resistant to temperature stresses. Deeper waters in these locations experience slightly cooler water temperatures in summer and higher winter water temperatures than shallow waters due to the presence of the Gulf Stream (Atkinson et al. 1983). *O. arbuscula* from one shallow location experience higher growth rates (Miller 1995) and increased heterotrophy when exposed to summer versus winter water temperatures (Aichelman et al. 2016). The response of deeper *O. arbuscula* is unknown, but these results indicate a physiological response to temperature changes that warrants further study. Here we experimentally investigated whether elevated temperatures differentially affected *O. arbuscula* conspecifics collected from shallow (less than nine meters) and deeper (greater than 20 meters) locations in terms of growth, bleaching, and composition of their microbiome and eukaryotic endosymbionts.

4.3 Methods

Oculina arbuscula was collected in August 2018 from natural and artificial reefs off Morehead City, North Carolina, USA - Radio Island Jetty (34-42'54" N , 076-41'06" W) and the Liberty Ship (34-40’21”N, 076-44’43”W) at less than nine meters deep and the Indra Ship (034-33’55”N, 076-58’30”W) and the Novelty Ship (34-39’29”N, 76-48’26”W) from their decks at greater than 20 meters deep. Twenty-five to 30 coral fragments were

randomly collected from separate colonies at each site using pliers. Fragments were marked by location of collection, placed in seawater-soaked bubble wrap, stored in coolers to prevent thermal shock, and transported to a 20-tank aquarium system (37L per tank, temp ~27.4°C, each tank independently recirculating) at the Georgia Institute of Technology in Atlanta, GA.

Each 37-L tank in the aquarium system contained one 50-watt neotherm heater that independently controlled temperature and one pump for continuous water circulation. Lighting was provided by banks of 4 tube lights; two were true actinic and two were T5 coral plus bulbs, providing a spectrum known to facilitate coral growth. They provided 210 μmol photons $m^{-2}s^{-1}$ to the bottom of the tank and cycled on a 12-hour light-dark cycle. This lighting regime maintained light levels at the lower end of light levels documented at one location (Radio Island Jetty) in previous tank experiments using the same species (Aichelman et al. 2016). Tank temperature (27 - 29°C depending on treatment, after an initial acclimation period at 27°C) and salinity (36-37 ppt) were monitored daily using Thermofisher Orion Star pH meter (relative accuracy ±0.01 units). Salinity was maintained via periodic addition of R/O freshwater as needed. Twenty percent water changes occurred twice weekly using saltwater made with Instant Ocean Reef Crystals. Corals were fed hatched *Artemia spp.* ad libitum once per week prior to the first water change of the week. Corals were monitored for epiphytic algal growth on dead portions of the coral daily, and any visible algae were removed via gentle scrubbing with a toothbrush.

Upon arrival at the lab, coral fragments were cut to 4-6 cm in length using a Dremel 4000 rotary tool. One fragment from each collection site was randomly assigned to each tank to avoid confounding tank and genotype (i.e., each tank contained 4 treatment corals,

with one being from each site). After this distribution, there were 3-4 fragments from each collection site remaining (13 total); these remaining fragments were randomly assigned to tanks (1 to each tank until no corals were remaining) and remained there through the end of the acclimation period (see below). These were then used to assess coral microbiome composition at the initiation of the experiment after acclimation to lab conditions (see below).

Fragments were acclimated to lab conditions at 27.4 0°C for four weeks. After this acclimation period, the excess (non-treatment, corals) were collected, placed in RNAlater and frozen at -80°C for microbiome analysis, and half of the tanks were randomly assigned to an elevated temperature treatment (29.0°C ±0.17) while the other tanks remained at ambient temperature (27.4°C ±0.12). Acclimation temperature mimicked average summer water temperatures at two buoys located close to collection sites off the coast of North Carolina (NOAA National Data Buoy Center – Station BFTN7 & 41159), while elevated temperatures were chosen to mimic predicted future temperature levels (Hoegh-Guldberg et al. 2014). Elevated temperature tanks were slowly ramped up over one week. The effect of temperature on coral growth, survivorship, and the composition of Symbiodiniaceae and microbiome was then determined over the course of an eight-week experiment.

Coral growth was measured at the start of the experiment, midway through the experiment (week 4), and at the conclusion of the experiment (week 8). Changes in skeletal growth were monitored using the buoyant weight method (Davies 1989). Corals were then shaken gently twenty times to remove excess water and total weight was measured (skeletal and tissue weight, Clements & Hay 2019). Changes in tissue growth were determined by

subtracting the skeletal weight from the total wet weight. Percent changes in coral growth over the course of the experiment were then evaluated.

PAM fluorometry was used as a proxy for coral health via evaluating the photosynthetic output of the coral's Symbiodiniaceae. PAM readings were taken once per week. The aquarium lights above the corals were turned off one hour prior to taking measurements so as to dark-adapt the corals. Three PAM readings were taken randomly over each coral and an average of those three readings was taken to be the average Fv/Fm for that coral fragment. At the end of the experiment, fragments collected from Radio Island Jetty were removed from PAM analysis as they lacked pigmentation from the on-set of the experiment (leading to a zero PAM reading) through the conclusion of the experiment and, thus, created no measurable variance within the population.

Corals were collected from the tanks for microbiome analysis at two points over the course of the experiment. A sub-set of corals (the non-treatment corals described above) were collected from the tanks post-acclimation and prior to experiment initiation. All remaining corals were collected at the end of the experiment. All corals were preserved in RNAlater immediately upon removal from the tanks and stored at -80°C until later analysis of microbiome and Symbiodiniaceae composition. Approximately 1 l of water from each tank also was filtered onto a 0.2 μm polyethersulfone (PES) filter and preserved in RNAlater to assess the microbiome of the water in each tank. A WaterPik was used to remove tissue from thawed corals (Johannes & Wiebe 1970), and this tissue slurry was centrifuged at 10,000 RPM for 10 minutes. The resulting tissue pellet was weighed, homogenized in 4 mL of sterile seawater using a tissue homogenizer, and vortexed. 2.0 mL of the resulting tissue slurry was preserved in 0.05% gluteraldeyde for endosymbiont

analysis while the remaining 2.0 mL was used for microbiome analysis (see below). Endosymbiont density was determined using a haemocytometer, and the average number of endosymbiont cells/gram of removed tissue was determined.

In preparation for microbiome analysis, two ml of tissue homogenate from each sample was transferred to a microcentrifuge tube and centrifuged at 13,000 rpm for five min. Both the resultant pellet and the 0.2 μm filter for each sample were added directly to PowerBead tubes from a PowerSoil DNA Isolation Kit (Qiagen) and DNA was extracted according to the manufacturer's instructions. Purified DNA was quantified using a Qubit 2.0 fluorometer (ThermoFisher Scientific).

For the bacterial component of the microbiome, the V3-V4 hypervariable region of the 16S rRNA gene was amplified using universal 16S rRNA gene primers 515F (Parada) (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (Apprill) (5'-GGACTACNVGGGTWTCTAAT-3'). For the Symbiodiniaceae component, the internal transcribed spacer 2 (ITS2) region of the nuclear ribosomal RNA was amplified using primers itsD (5'-GTGAATTGCAGAACTCCGTG-3') and its2rev2 (5'-CCTCCGCTTACTTATATGCTT-3') (Cunning 2017, Stat 2009). Primers were modified with sample-specific barcode sequences and Illumina-sequencing adapters according to Kozich et al. (2013).

For each sample, 1.5 μl of DNA (total reaction volume of 25 μl) was amplified using GoTaq DNA Polymerase (ThermoFisher Scientific) with a final primer concentration of 0.2 μM and 10 μg of bovine serum albumin (BSA; New England Biolabs, Inc.) added as a PCR enhancer. For bacterial samples, PCR cycling conditions were an initial

denaturation at 95°C for 3 min, followed by 30 cycles of a 45-s denaturation step (95°C), 45-s primer annealing step (55°C), and 90-s extension step (72°C), with a final extension step of 10 min at 72°C.

For Symbiodiniaceae samples, PCR cycling conditions were an initial denaturation at 95°C for 3 min, followed by 30 cycles of a 45-s denaturation step (95°C), 45-s primer annealing step (57°C), and 90-s extension step (72°C), with a final extension step of 10 min at 72°C. PCR products were run on a 1% agarose-Tris-acetate-EDTA (TAE) gel along with no DNA negative controls to verify amplicon size and the absence of contamination. The products were purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and quantified using the Qubit 2.0 fluorometer. Equimolar concentrations of each amplicon were pooled, mixed with 10% PhiX DNA to increase template diversity and sequenced using a 500-cycle paired-end MiSeq reagent V2 kit on an Illumina sequencer.

After demultiplexing, barcoded sequences were trimmed and filtered using Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/, Phred score > 25, minimum sequence length >100 nt). Paired-end sequences were merged and exact sequence variants (ESVs) were determined from filtered sequences using DADA2 in the QIIME2 pipeline (Callahan 2016, Bolyen et al., 2019). For bacterial sequences, taxonomy was assigned to ESVs by comparison to the SILVA ribosomal RNA database (Release 132). For Symbiodiniaceae sequences, taxonomy was assigned by comparison to a custom-built database as described in Johnston et al (2020). Singletons and ESVs assigned to chloroplast, mitochondria, or coral sequences were removed from further analyses. Alpha- and beta-diversity metrics were calculated in the R package vegan (v2.5-6) and plotted in the R package phyloseq (v.1.28.0) (McMurdie 2013).

Data were analyzed using the R statistical software version 3.6.0 using the car 2.1-5 (Fox & Weisberg 2019), lme4 1.1-21 (Bates et al. 2015), and emmeans 1.4 (Lenth 2019) statistical packages. The effects of temperature on endosymbiont Fv/Fm values over time, endpoint endosymbiont density, and coral growth were analyzed using linear mixed models to include tank as a random factor and collection site as a blocking factor. Tukey posthoc analysis was conducted on tissue growth and total growth. Initial endosymbiont densities were evaluated using a generalized linear model.

4.4 Results

Coral growth differed between treatments. Buoyant weight was not significantly affected by temperature or depth (Figure 4.1A). There was a significant temperature x depth interaction that affected coral tissue growth (Figure 4.1B, Temperature*Depth $p=0.028$). Corals from deeper sites grew 8.3% at 27.4 ºC, but loss 5.0% of their tissue mass at 29ºC. Shallow water corals lost 1.9-3.0% of their tissue under both temperature treatments. There was also a significant depth x temperature interaction for total growth (Figure 4.1C, $p=0.028$). Deep water corals grew 5.2% at 27.4ºC, but they lost 0.5% of their total mass at 29ºC. Shallow coral mass increased 0.7% at 27.4ºC but decreased 0.5% at 29ºC.

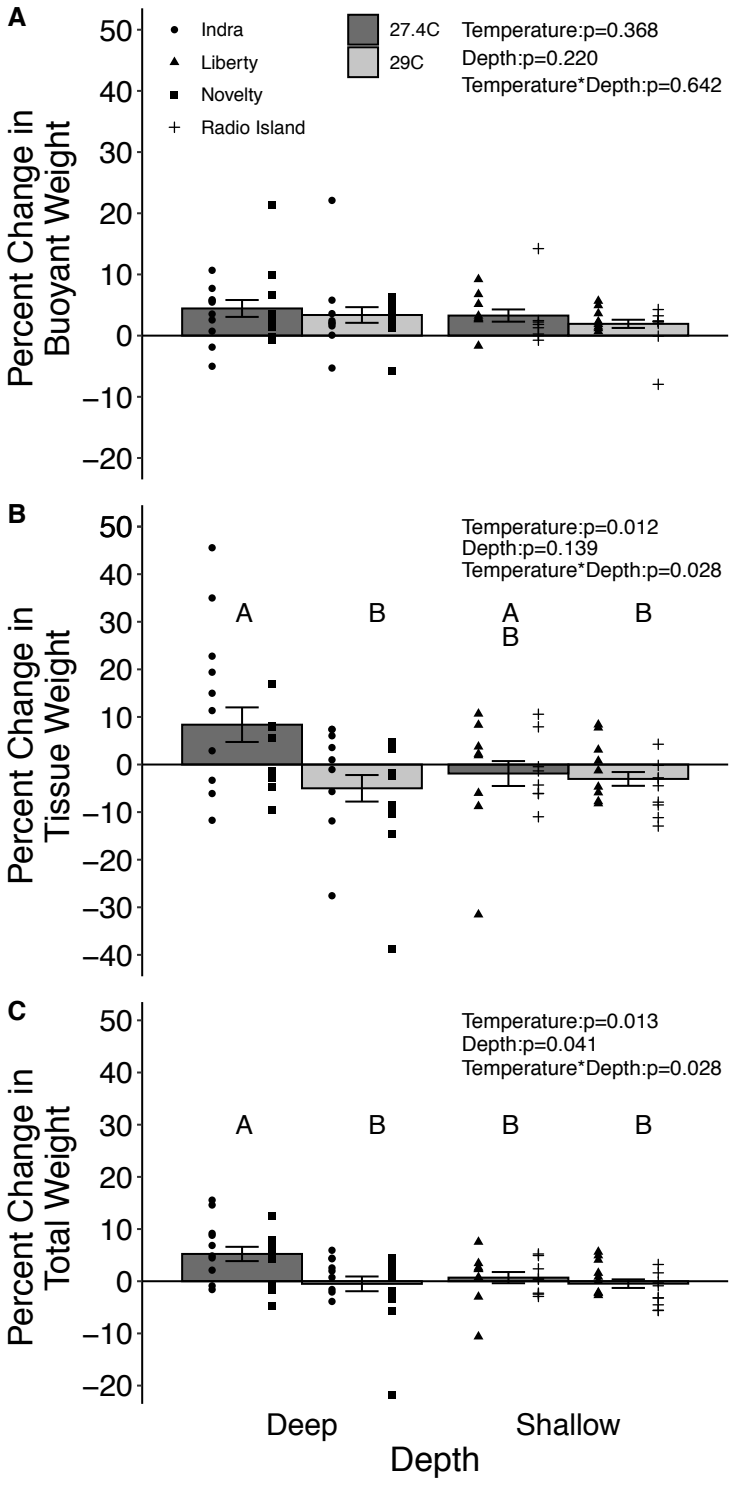

Figure 4.1: The effect of elevated temperature levels on A) Buoyant weight (skeletal growth), B) Tissue Growth, and C) Total Growth (tissue + skeleton) across the two depths. Growth was measured as change in weight over time. Data analyzed using linear mixed models. Error bars represent standard error. Dots represent individual data points. Shapes represent location. Letters represent treatments that are significantly different based on posthoc analysis.

Coral microbiomes did not differ significantly in species composition due to depth (PERMANOVA, R: 0.014 p=0.470), collection site (PERMANOVA, R: 0.022 p=0.636), or temperature treatment (PERMANOVA, R: 0.016 p=0.358) (Figure 4.2A). Microbiome compositional variability did not differ due to depth (PERMDISP2, p=0.079), temperature, (PERMDISP2, p=0.710), or collection site (PERMDISP2, Liberty Ship-Indra Ship p=0.255, Novelty Ship-Indra Ship p=0.999, Radio Island-Indra Ship p=0.994, Novelty Ship-Liberty Ship p=0.259, Radio Island-Liberty Ship p=0.529, Radio-Island-Novelty Ship p=0.998). Additionally, no significant differences were seen in species diversity in either depth (ESVs richness, p=0.804, Shannon diversity, p=0.986), temperature (ESVs richness, p=0.916, Shannon diversity, p=0.738), or collection site (Liberty Ship-Indra Ship ESVs p=0.744 Shannon p=0.982, Novelty Ship-Indra Ship ESVs p=0.603 Shannon p=0.882, Radio Island-Indra Ship ESVs p=0.494 Shannon p=0.868, Novelty Ship-Liberty Ship ESVs p=0.824 Shannon p=0.901, Radio Island-Liberty Ship ESVs p=0.671 Shannon p=0.886, Radio Island-Novelty Ship ESVs p=0.824 Shannon p=0.983).

Coral microbiomes did differ significantly from surrounding tank water (PERMANOVA, R: 0.188 p<0.001) (Figure C1). The microbiome of the water was generally more diverse with a higher proportion of reads mapping outside of the top fifteen most abundant families compared to their respective coral counterparts (Figure 4.2B). Bacterial taxa in the family Flavobacteriaceae were more common in water samples ranging from 15 – 50% of the total community but averaging only ~6% of coral microbiomes. Water microbiomes had very few sequences mapping to members of the Gammaproteobacteria, specifically the families Alcanivoracaceae, Marinobacteraceae,

Pseudomonadaceae, Alteromonadaceae, and the Oceanospirillales family SS1-B-06-26. These families accounted for 50 – 80% of coral bacterial communities but <4% of water microbiomes.

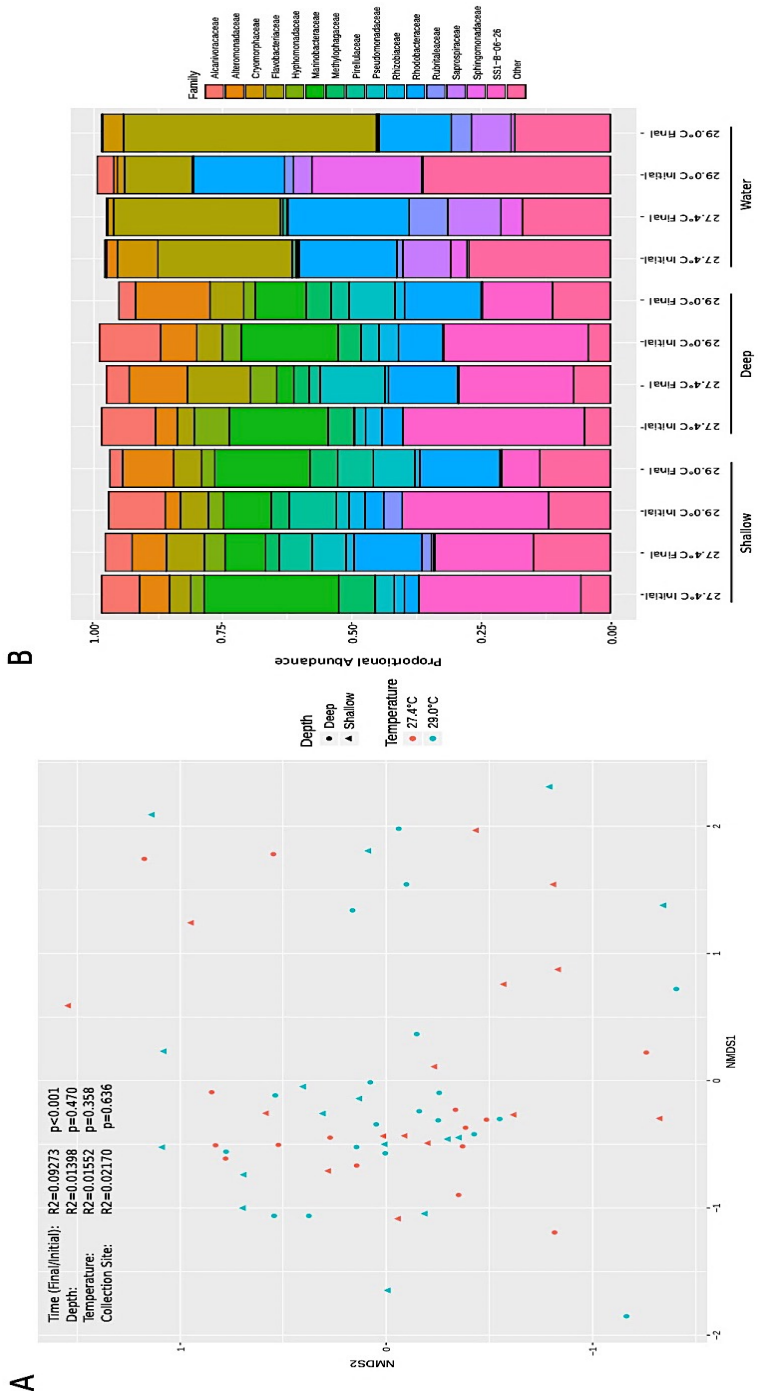

Figure 4.2: (A) NMDS plot of bacterial microbiome composition (Bray-Curtis dissimilarity) of coral samples (B) Proportional representation of the top fifteen most abundant bacterial families. All other families grouped in to the Other category.

There was a significant collection site x depth interactive effect on endosymbiont density at the initiation of the experiment (Figure 4.3A, p=0.038). Corals collected from sites other than Radio Island contained 22-24x, on average, the number of endosymbionts as corals collected from the shallow Radio Island site. At the end of the experiment, collection site no longer interacted significantly with depth of collection (Depth*Location: p=0.733), but depth and temperature both significantly impacted final endosymbiont density (Temperature: p<0.001;Depth:p=0.050) with a marginally non-significant interactive effect (p=0.082). Higher temperatures led to a 54% (shallow sites) to 73% (deep sites) decrease in endosymbiont density. Corals from shallow sites possessed 17% (29 ºC) to 52% (27.4 ºC) fewer endosymbionts than corals from deeper sites.

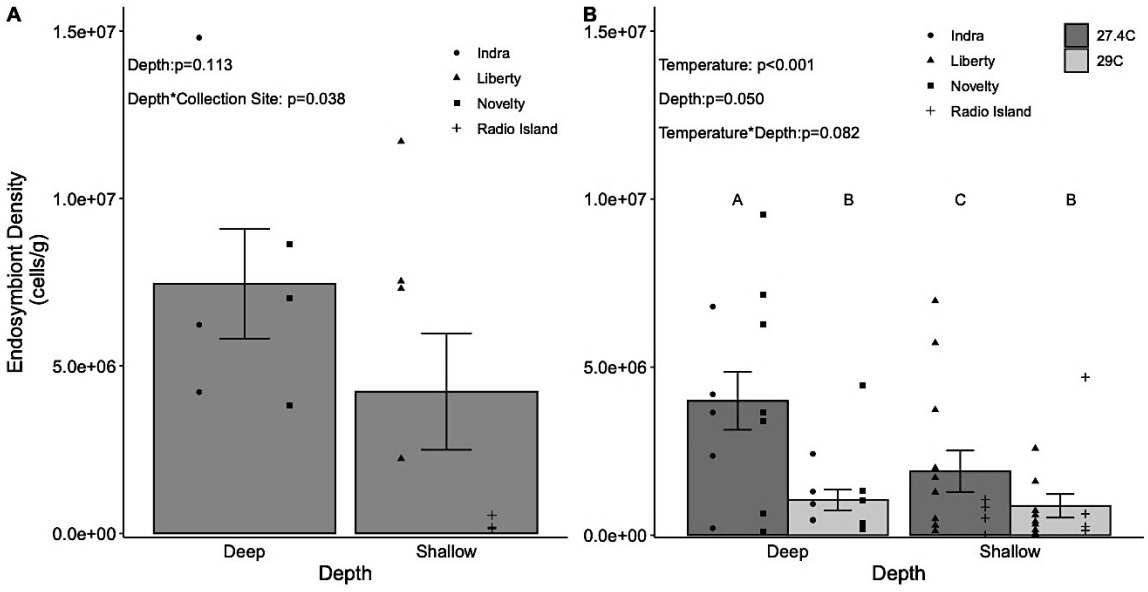

Figure 4.3: Endosymbiont densities at each depth and location. (A) Initial densities, (B) Final densities. Initial density analyzed using generalized linear model and final density analyzed using linear mixed models. Error bars represent standard error. Dots represent individual data points with each shape representing a different location.

Endosymbiont photosynthetic efficiency, measured as Fv/Fm, decreased across all treatments over the course of the eight-week experiment (Figure 4.4,Week: $p<0.001$). Fv/Fm, values decreased 30-35% during the eight-week experiment for corals at 27.4 ºC, but the decrease was 55-78% for corals at 29 ºC. At week eight, photosynthetic efficiency of corals at 29ºC was 33-67% lower than for corals at 27.4 ºC ($p<0.001$).

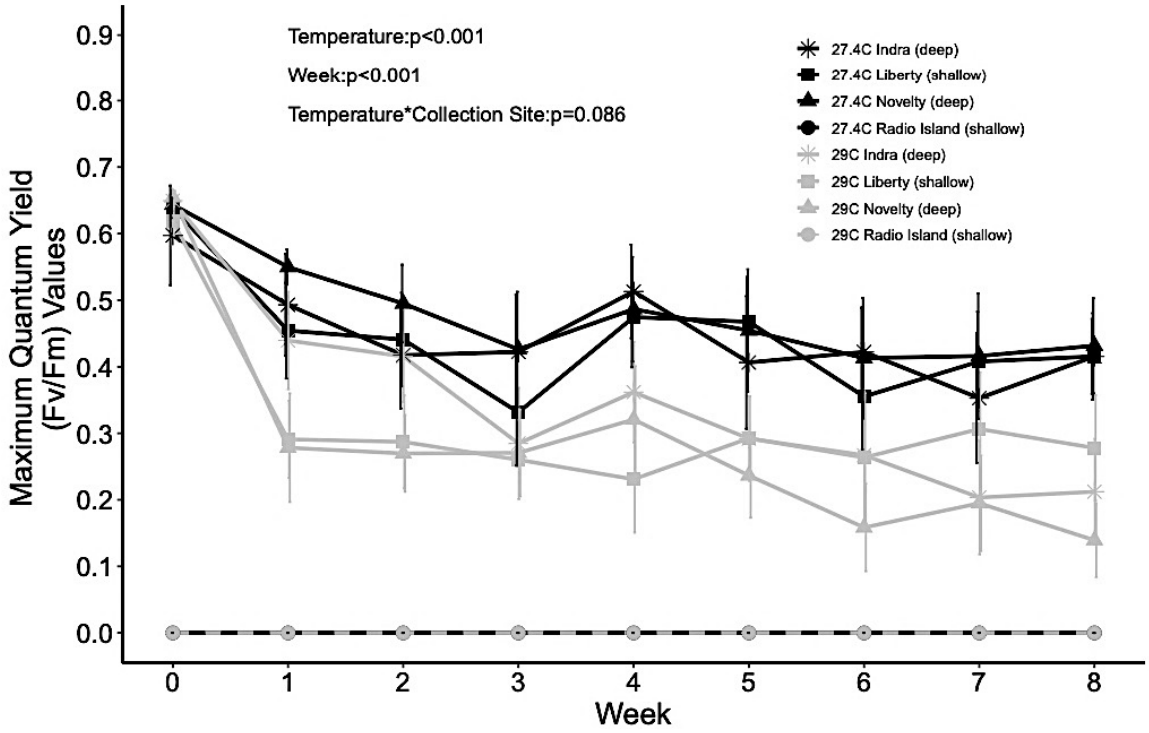

Figure 4.4: Effect of temperature and collection site on Fv/Fm values over time. Data analyzed using linear mixed models. Bars represent standard error. Radio Island data not included in final analysis due to lack of variance in dataset.

Since Symbiodiniaceae sequences were difficult to amplify from the water samples, no comparison was done between coral and water samples. In contrast to coral

microbiomes, Symbiodiniaceae composition did differ significantly due to depth (PERMANOVA, R: 0.046 p=0.019) and nearly for temperature (PERMANOVA, R: 0.035 p=0.057) but not for collection site (PERMANOVA, R: 0.028 p=0.403) (Figure 4.5A). Similarly, compositional variability differed significantly due to depth (PERMDISP2, p=0.032) but not for temperature (PERMDISP2, p=0.147) or collection site (PERMDISP2, Liberty Ship-Indra Ship p=0.606, Novelty Ship-Indra Ship p=0.999, Radio Island-Indra Ship p=0.467, Novelty Ship-Liberty Ship p=0.518, Radio Island-Liberty Ship p=0.963, Radio Island-Novelty Ship p=0.403). There were also significant differences in Symbiodiniaceae ESV diversity for corals collected at different depths (ESVs richness, p=0.044, Shannon diversity, p=0.233) but not for temperature (ESVs richness, p=0.281, Shannon diversity, p=0.608) or the remainder of collection sites (Liberty Ship-Indra Ship ESVs p=0.170 Shannon p=0.586, Novelty Ship-Indra Ship ESVs p=0.643 Shannon p=0.759, Radio Island-Indra Ship ESVs p=0.390 Shannon p=0.353, Novelty Ship-Liberty Ship ESVs p=0.072 Shannon p=0.386, Radio Island-Liberty Ship ESVs p=0.924 Shannon p=0.667, Radio Island-Novelty Ship ESVs p=0.243 Shannon p=0.223). All samples were dominated by a single ESV, with 100% sequence identity to *Breviolum psygmophilum*, accounting for upwards of 90% of the total community (Figure 4.5B). Of the minor members of the Symbiodiniaceae community, only one ESV showed enrichment under any comparison. ESV3 (99.28% sequence identify to *Breviolum psygmophilum*) was enriched under ambient conditions (Figure 4.6; p=0.002).

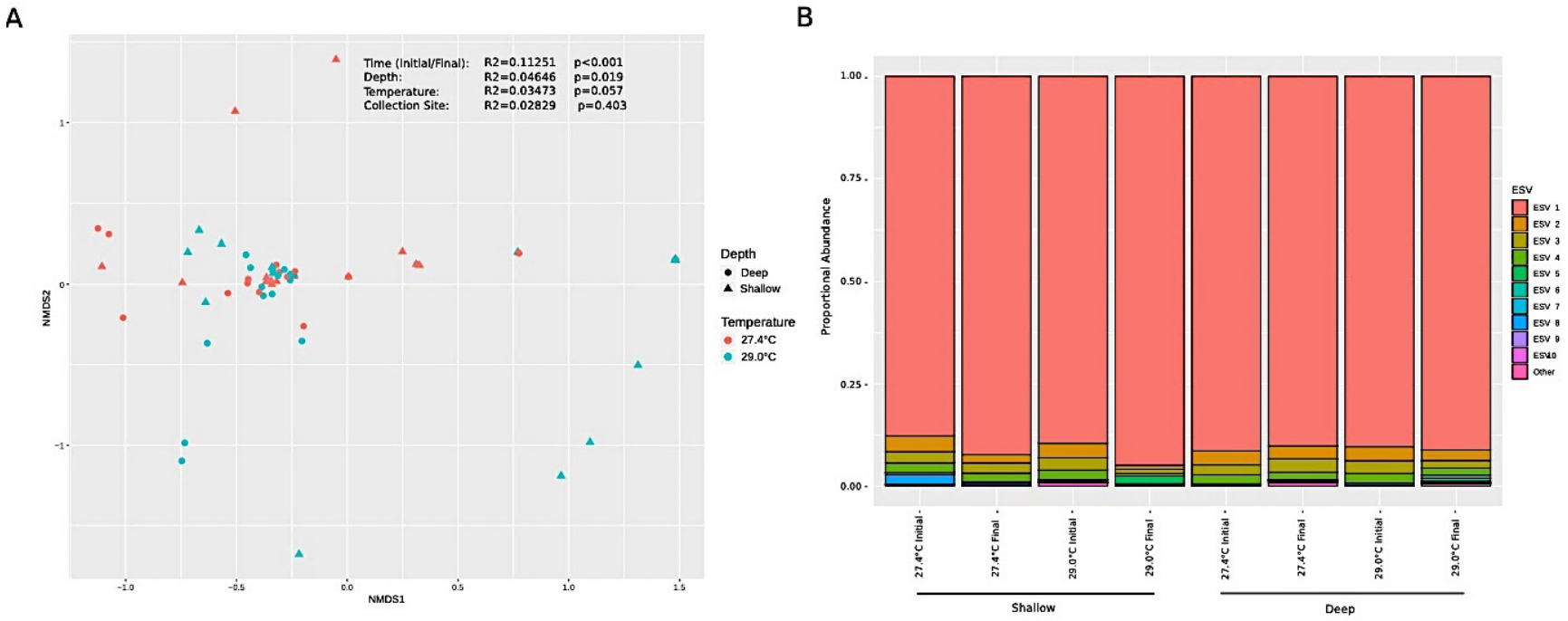

Figure 4.5: (A) NMDS plot of Symbiodiniaceae composition (Bray-Curtis dissimilarity) of coral samples (B) Proportional representation of the op ten most abundant Symbiodiniaceae ESVs. All other ESVs grouped into the Other category.

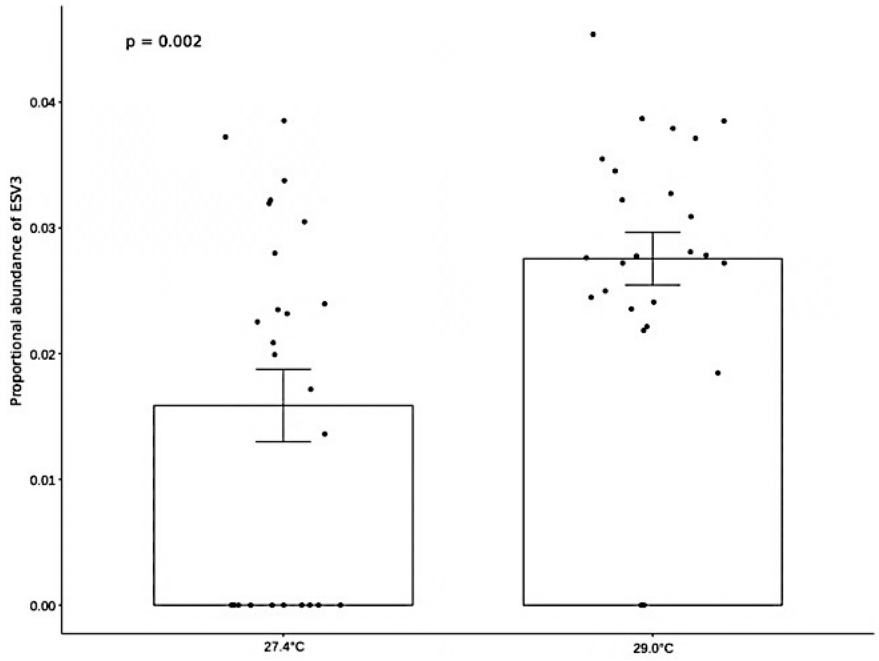

Figure 4.6: Proportional abundance of Symbiodiniaceae ESV3 under 27.4°C and 29°C conditions.

4.5 Discussion

Oculina arbuscula exhibited reduced endosymbiont density and photosynthetic efficiency in response to elevated temperature, but individuals from the deeper sites experienced greater reductions. At 27.4°C, tissue growth of *O. arbuscula* from deeper reefs was 8.3% versus a 5.0% loss in tissue mass when grown at 29°C. Total growth (tissue mass plus buoyant weight) exhibited a similar pattern, increasing by 5.2% at ambient temperature and declining by 0.5% at the elevated temperature for corals from deeper sites. Corals from shallow sites grew minimally during our 8-week experiment without differing significantly in buoyant weight, tissue growth, or total growth as a function of temperature (Figure 4.1). Change in buoyant weight of shallow corals was 3.3% and 1.9% for corals at ambient and elevated temperatures, respectively; tissue growth was -1.9% and -3.0%,

producing a total growth change of 0.7% and -0.5%, which made total growth undetectable under these conditions.

Shallow, near-shore environments are often more turbid locations with greater nutrient input and larger fluctuations in temperature and carbonate chemistry, while deeper waters exhibit more stable environments (Camp et al. 2018). Corals found in stressful environments can exhibit greater physiological plasticity, which may enhance their tolerance to climate stressors (Camp et al. 2018). This acclimation to variable environments may come with a trade-off (Anthony & Connolly 2004) as corals from shallow sites failed to grow significantly even at 27.4°C.

The specific adaptation or acclimation strategy that allows shallow corals to survive in more variable environments and withstand temperature stress was not explicitly studied, but hypotheses can be proposed. Genotype and microbiome or Symbiodiniaceae composition can impact coral resilience (Barshis et al. 2013, Bay & Palumbi 2014, Ziegler et al. 2017). Genotype was not studied in this experiment, and relatedness between *O. arbuscula* across collection sites is unknown. We did however evaluate composition of the symbionts and the microbiome members associated with deep and shallow corals. Unlike other studies that have documented a relationship between microbiome and temperature-induced coral bleaching in *Oculina patagonica* (Kushmaro et al. 1997, Kushmaro et al. 2001, Mills et al. 2013), microbiome composition did not differ significantly between temperature treatments or depth collection sites for *O. arbuscula* (Figure 4.2). However, endosymbiont density did differ between depths (Figure 4.3) and could have played a role in generating growth rate differences. These differences in endosymbiont density may have been linked to the differences in light availability at the deeper collection sites compared

to the nearshore sites. Greater light availability enhances *O. arbuscula* growth (Miller 1995 – measured as total wet mass). Previous research found higher growth rates, likely due to greater light availability, at shallower depths (Miller 1995), however this research only considered corals collected from one site (Radio Island Jetty) at depths less than six meters. Our results are also suggestive of a positive effect of light on coral growth as offshore waters of the North Atlantic Bight are often less turbid, with greater light availability. Although not directly studied in our experiment, coral growth from these sites may further be aided by the lack of algal competition found on artificial reefs that naturally enhances coral growth compared to shallow sites and natural deep-water rocky reefs with greater algal presence (Miller & Hay 1996).

It is plausible that shallow corals adapt to more stressful environments and lower light availability by lowering their endosymbiont density relative to their deeper conspecifics. This lowered endosymbiont density may create a growth tradeoff as photoautotrophic carbon acquired from the endosymbionts becomes unavailable (Hughes et al. 2010). While *O. arbuscula* rely on endosymbiont-derived nutrients when they possess the symbionts, aposymbiotic colonies can survive on heterotrophy alone (Leal et al. 2014). Heterotrophy can mitigate tissue and skeletal losses for bleached corals in some species (Aichelman et al. 2016, Hughes et al. 2010). Thus, *O. arbuscula* in shallow, turbid, environments with more flow may rely primarily on heterotrophy instead of photosynthesis of endosymbionts. Our periodic feedings in the lab may have resulted in less input from heterotrophy than occurs in the wild, allowing heterotrophically acquired carbon to sustain skeletal growth but not measurable tissue growth.

Across treatments, corals exhibited reductions in photosynthetic efficiency over the course of the experiment – likely due to acclimation to tank conditions. Although growth of corals from shallow areas was less significantly affected by temperature stress and shallow corals possessed, on average, fewer endosymbionts than deeper conspecifics, shallow corals still responded to elevated temperatures. Corals from both depths possessed 54-73% fewer endosymbionts and exhibited a 33-67% reduction in photosynthetic efficiency at 29°C than at 27.4°C (Figures 4.3&4.4). These results stand in contrast to corals found in nearshore, turbid environments on the Great Barrier Reef that exhibited reduced bleaching compared to offshore corals (Morgan et al. 2017). These discrepancies may be due to species-specific responses to thermal stress (Abrego et al. 2008). It is possible that there is not strong natural selection to avoid bleaching in *O. arbuscula* since they can survive and grow in an aposymbiotic state (Leal et al. 2014). Thus, loss of coloration in these corals, while indicative of an effect of elevated temperatures on these corals, may not lead to the high rates of mortality seen in corals that require endosymbionts for survival.

Although corals across both depths exhibited significant reductions in endosymbiont density and photosynthetic efficiency in response to elevated temperature, there was not a clear relationship between photosynthetic efficiency and endosymbiont density across collection sites. The two shallow sites possessed similar endosymbiont densities despite one site, Radio Island, exhibiting no photosynthetic efficiency over the course of the experiment (Figures 4.3,4.4). Further, the shallower Liberty Ship possessed significantly fewer endosymbionts than the two deeper collection sites despite exhibiting similar Fv/Fm values (Figure 4.4). These results may be due to variations in the photo-

physiology of Symbiodiniaceae. in corals from different collection sites. Differences in photo-physiology and thermal tolerance have been tied to Symbiodiniaceae genotype (Kemp et al. 2010, Ragni et al. 2010). Unlike those studies, the dominant endosymbiont species did not differ among collection sites or based on coral health (as measured as Fv/Fm – Figure 4.4). Corals across depth and temperature treatments possessed the same endosymbiont (*Breviolum psygmophilum* - LaJeunesse et al. 2012, 2018) at >80% relative abundance (Figure 4.5). Rather, it is possible that differences in the rare endosymbionts explain the discrepancies in photophysiology of Symbiodiniaceae in corals from different depths and collection sites. Endosymbiont ESVs from corals collected at shallow sites exhibited lower alpha diversity and greater beta-dispersion than their deep-water conspecifics (Figure 4.5). These rare endosymbionts may also play a role in the susceptibility of the coral to thermal stress (Ziegler et al. 2016). Temperature had a nearly significant impact on Symbiodiniaceae community composition ($p = 0.057$; Figure 4.5), and ESV3 was reduced by 35% in corals exposed to temperature stress (Figure 4.6). Rare endosymbionts may have a larger impact in the photophysiology and thermal tolerance of corals than is generally appreciated.

The similar reductions in photosynthetic efficiency and endosymbiont density for corals from the two depths make it reasonable to question the ability of deep-water reefs to serve as refugia for shallow corals under future warming regimes. Field studies comparing coral bleaching found corals from deeper reefs (11-30 meters deep depending on the study) were less impacted by bleaching than shallow conspecifics because of naturally cooler water temperatures on the deeper reefs (Penin et al. 2007, Smith et al. 2014, Muir et al. 2017). However, deeper reefs will likely be exposed to higher temperatures than those

currently experienced as the planet continues to warm (Barnett et al. 2005). At some point, deeper corals will likely also be affected by temperature stress (Gori et al. 2016). Our results mirror other research that found deep water corals to be significantly negatively affected by temperature stress (Spencer et al. 2000, Gori et al. 2016, Smith et al. 2016).

Some corals can quickly (in less than two years) acclimatize to a new, stressful environment (Jones et al. 2008, Bay & Palumbi 2015), and this may be a possibility for *O. arbuscula.* Although *O. arbuscula* corals from deeper waters possessed higher endosymbiont densities and tissue growth than shallow corals at 27.4°C, corals from both depths exhibited similar endosymbiont densities, photosynthetic efficiencies, and tissue loss at 29°C. This may be an indication of deep-water coral acclimatization that will assist *O. arbuscula* during a future facing elevated temperatures.

O. arbuscula studied in this experiment was collected from two distinct depths located within kilometers of each other. Despite the close proximity, corals from deeper waters exhibited significant reductions in growth rate when subjected to elevated temperature compared to their shallow conspecifics, although corals from both depths exhibited reductions in photosynthetic efficiency and endosymbiont density. These results question both the ability for deep-water reefs to serve as long-term refugia for corals and the possibility that corals from shallow, more variable environments are more resistant to prolonged thermal stress. Although temperate corals with facultative Symbiodiniaceae are not as dependent on their endosymbionts, these results suggest that temperate corals with facultative relationships are not less susceptible to thermal stress than corals with obligate endosymbionts. A coral's ability to resist elevated temperatures is not dependent solely on species identity, relationship with its endosymbiont, or depth. Rather a mixture of these

factors can lead to resistance, and it may be difficult to accurately predict which species and/or populations are most likely to survive a changing climate.

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APPENDIX A

Supplemental figures for Chapter 1

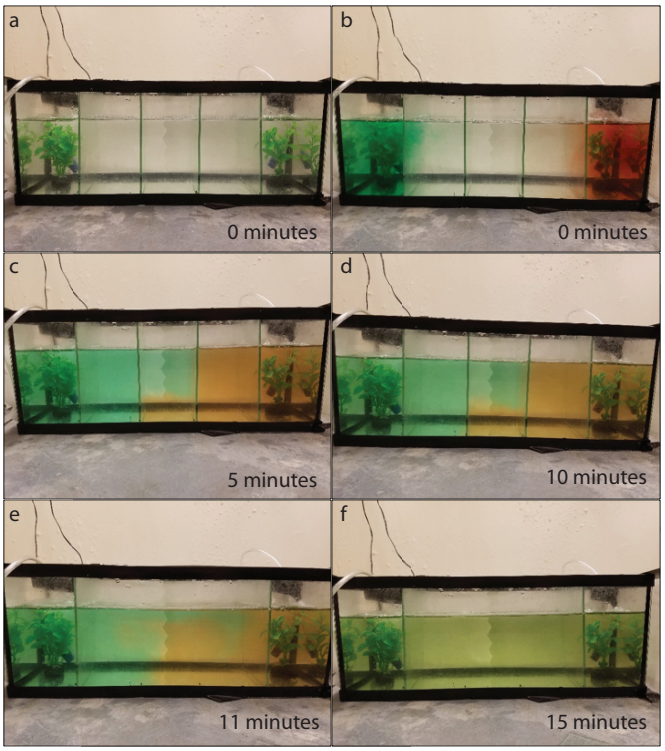

Fig. A1: Dye tests demonstrating the mixing of chemical cues produced by outer source compartments throughout the experimental period. A experimental tank before dye was added, glass panes in place to separate test fish during habituation period; B experimental tank immediately after dye was added to outer source compartments, glass panes in place to separate test fish during habituation period; C experimental tank after 5 min, glass panes in place to separate test fish during habituation period; D experimental tank after 10 min, glass panes in place to separate test fish during habituation period; E experimental tank after 11 min, glass panes removed from the tank, allowing test fish to move throughout the test space; and F experimental tank after15 min, conclusion of experiment

APPENDIX B

Supplemental figures for Chapter 3

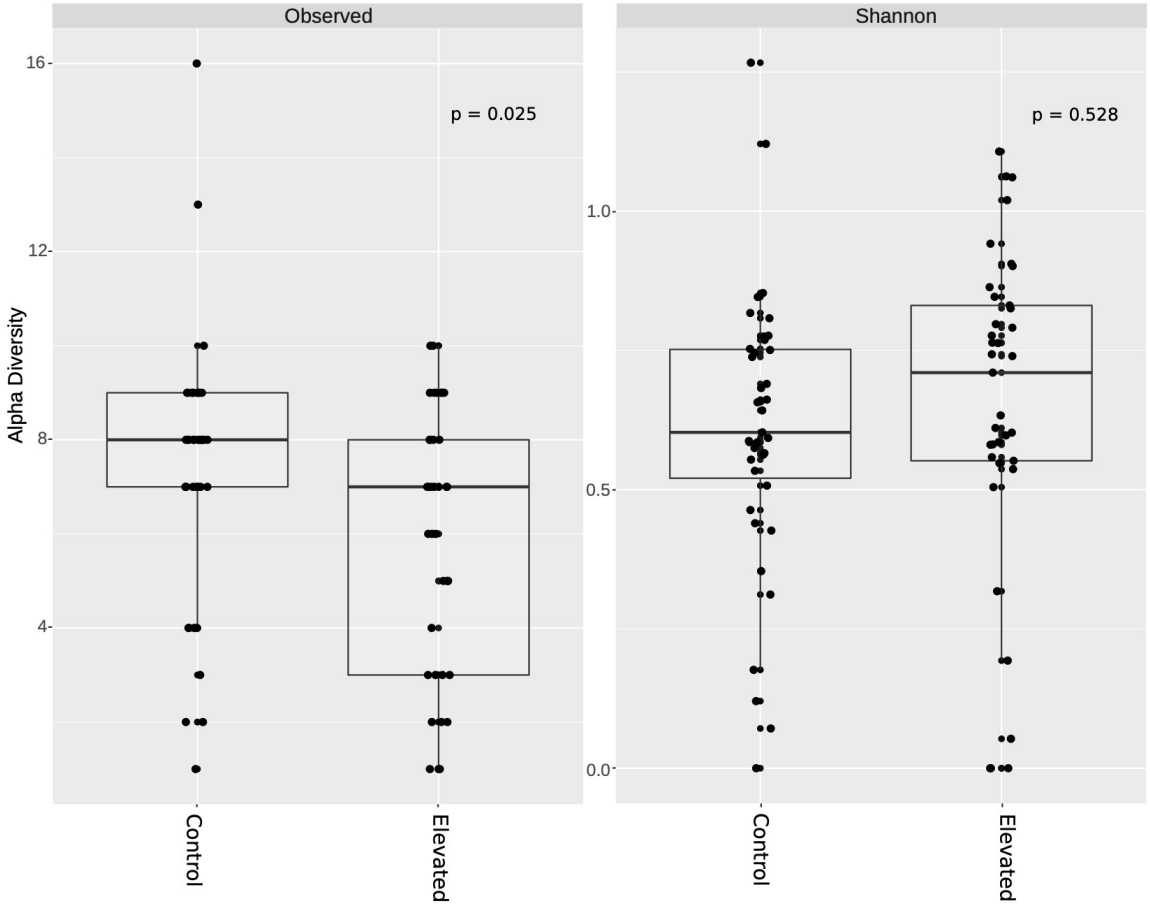

Figure B1: Alpha diversity of endosymbiont communities grouped by treatment type. The boxplot represents the 25th (lower) and 75th (upper) percentiles with the black line representing the median and dots representing individual data points.

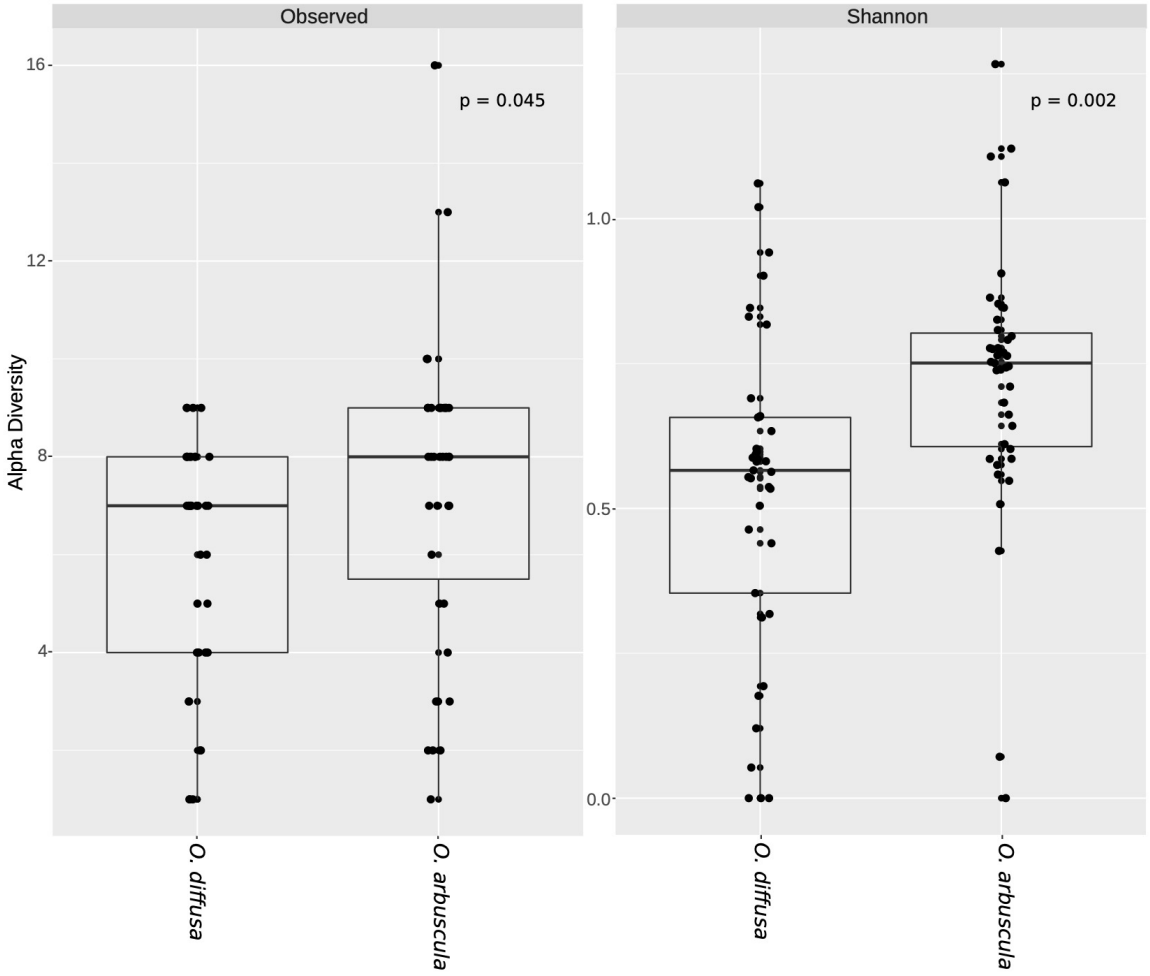

Figure B2: Alpha diversity of endosymbiont communities grouped by coral species. The boxplot represents the 25th (lower) and 75th (upper) percentiles with the black line representing the median and dots representing individual data points.

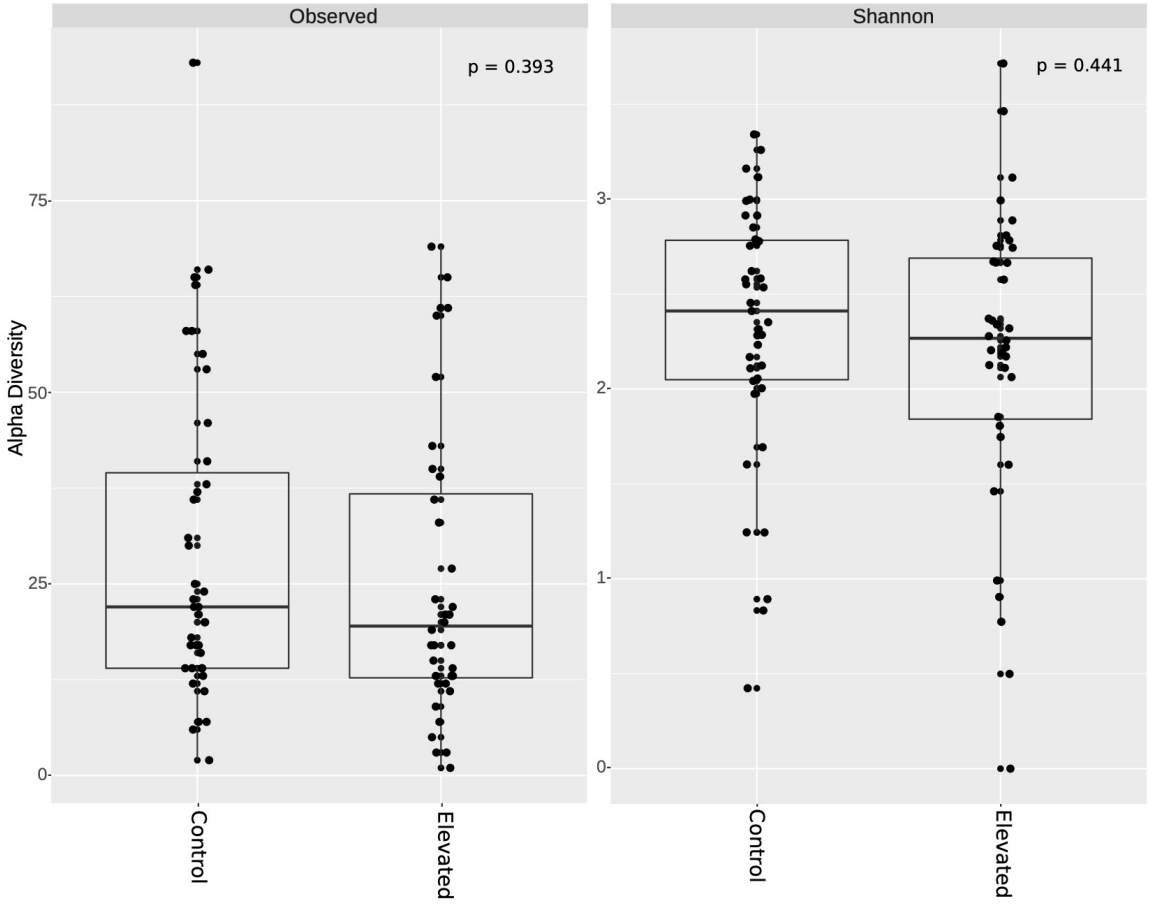

Figure B3: Alpha diversity of bacterial microbiomes grouped by treatment type. The boxplot represents the 25th (lower) and 75th (upper) percentiles with the black line representing the median and dots representing individual data points.

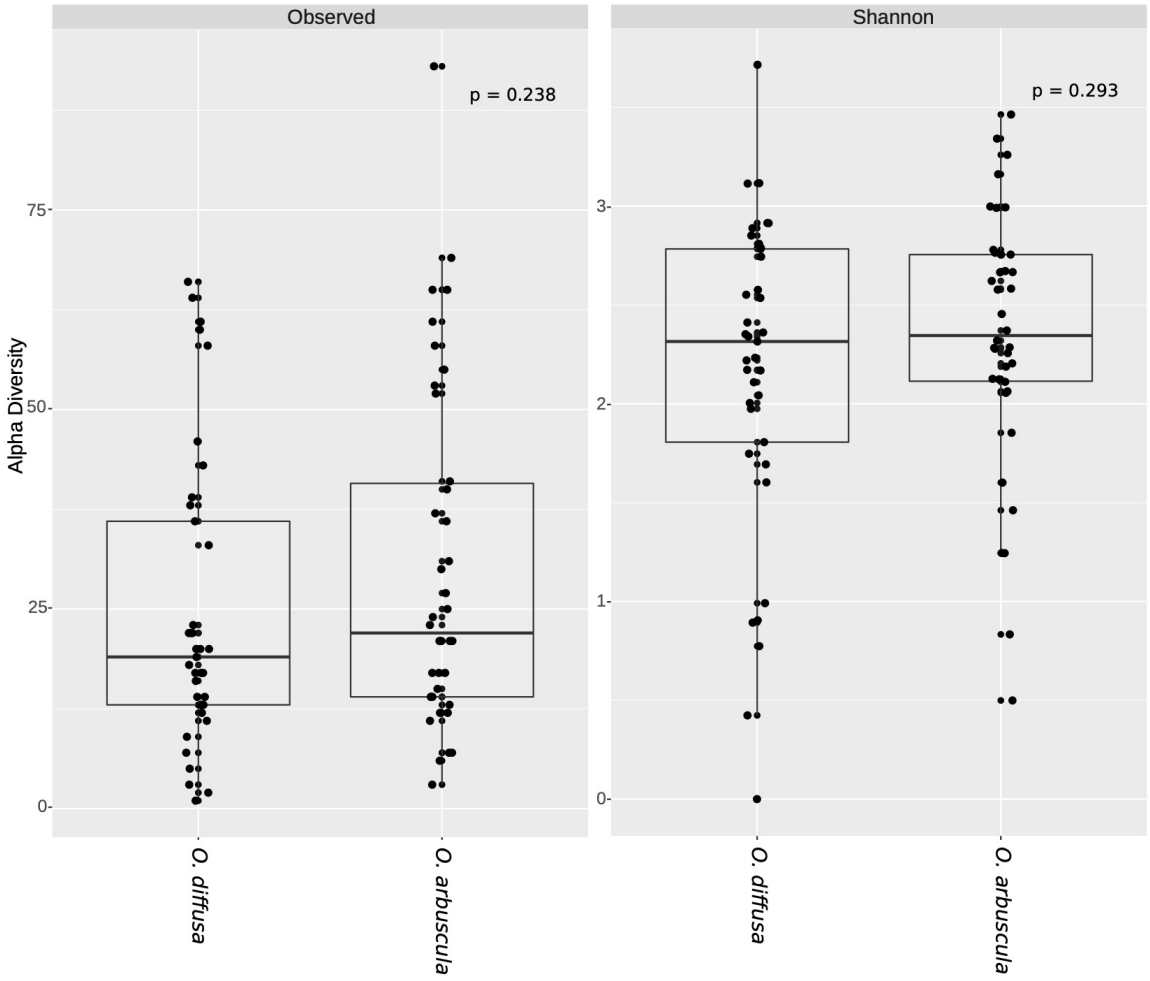

Figure B4: Alpha diversity of bacterial microbiomes grouped by coral species. The boxplot represents the 25th (lower) and 75th (upper) percentiles with the black line representing the median and dots representing individual data points.

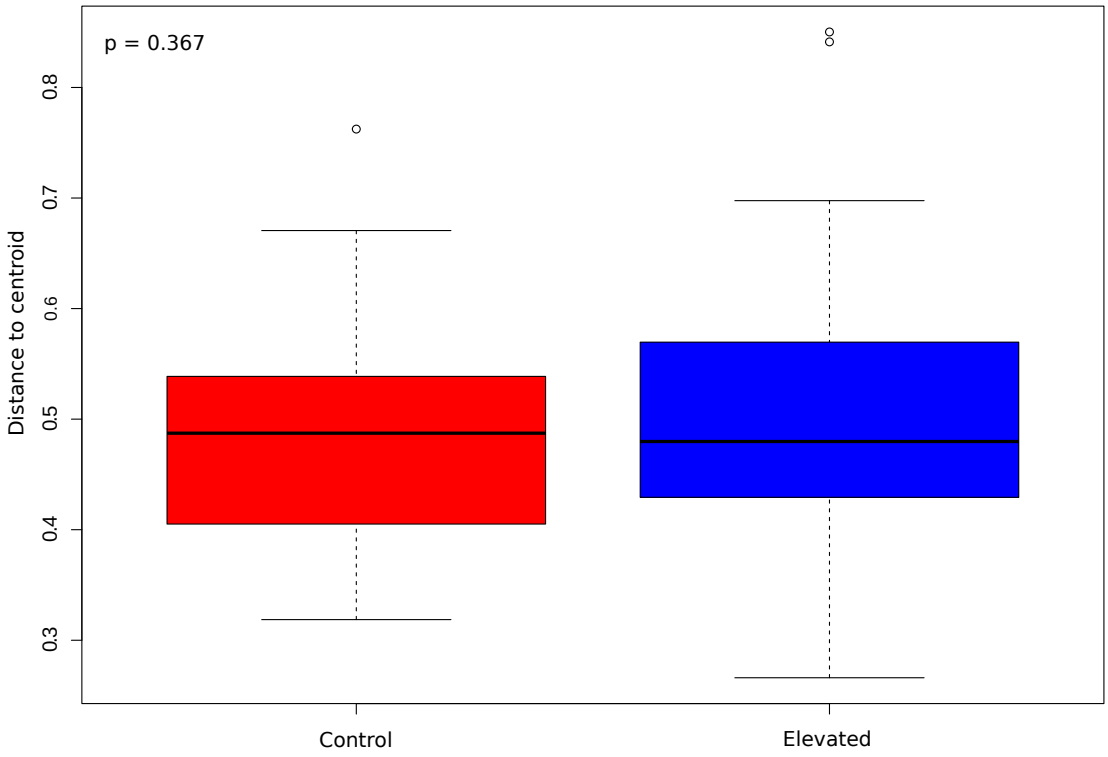

Figure B5: Distance to the centroid or betadispersion of bacterial microbiomes beta diversity (Bray-Curtis) grouped by treatment type. The boxplot represents the 25th (lower) and 75th (upper) percentiles with the black line representing the median. The whiskers are the interquartile range (IQR) with outliers represented by open circles.

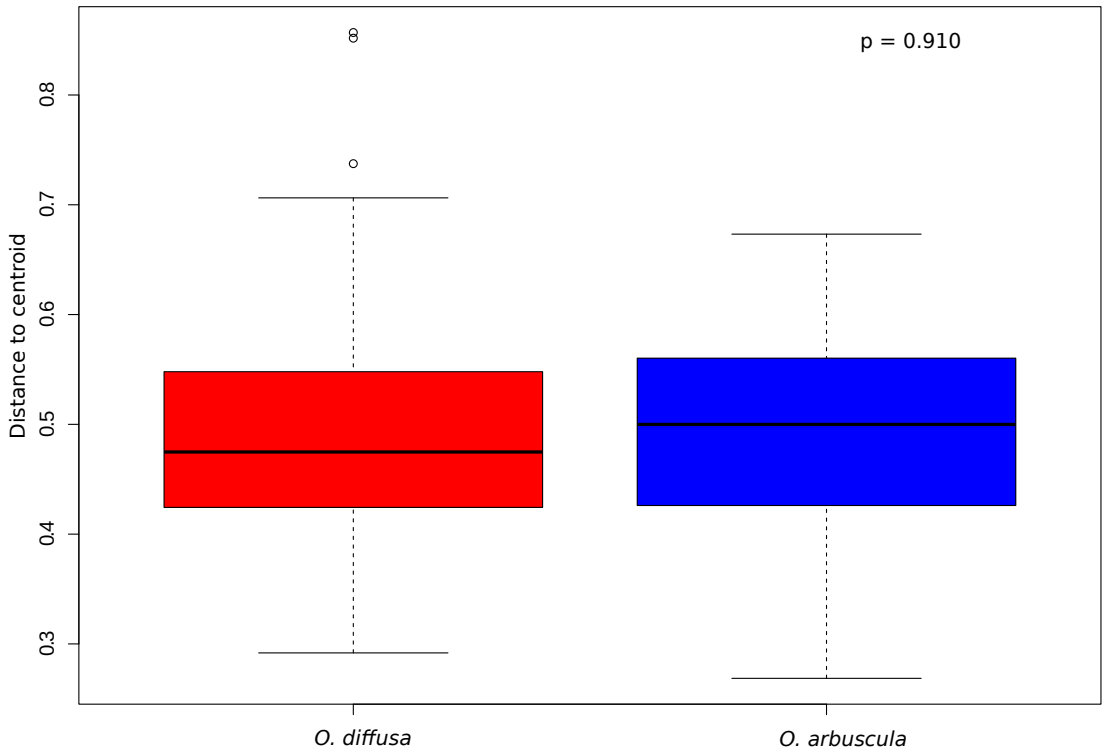

Figure B6: Distance to the centroid or betadispersion of bacterial microbiomes beta diversity (Bray-Curtis) grouped by coral species. The boxplot represents the 25th (lower) and 75th (upper) percentiles with the black line representing the median. The whiskers are the interquartile range (IQR) with outliers represented by open circles.

APPENDIX C

Supplemental Figures for Chapter 4

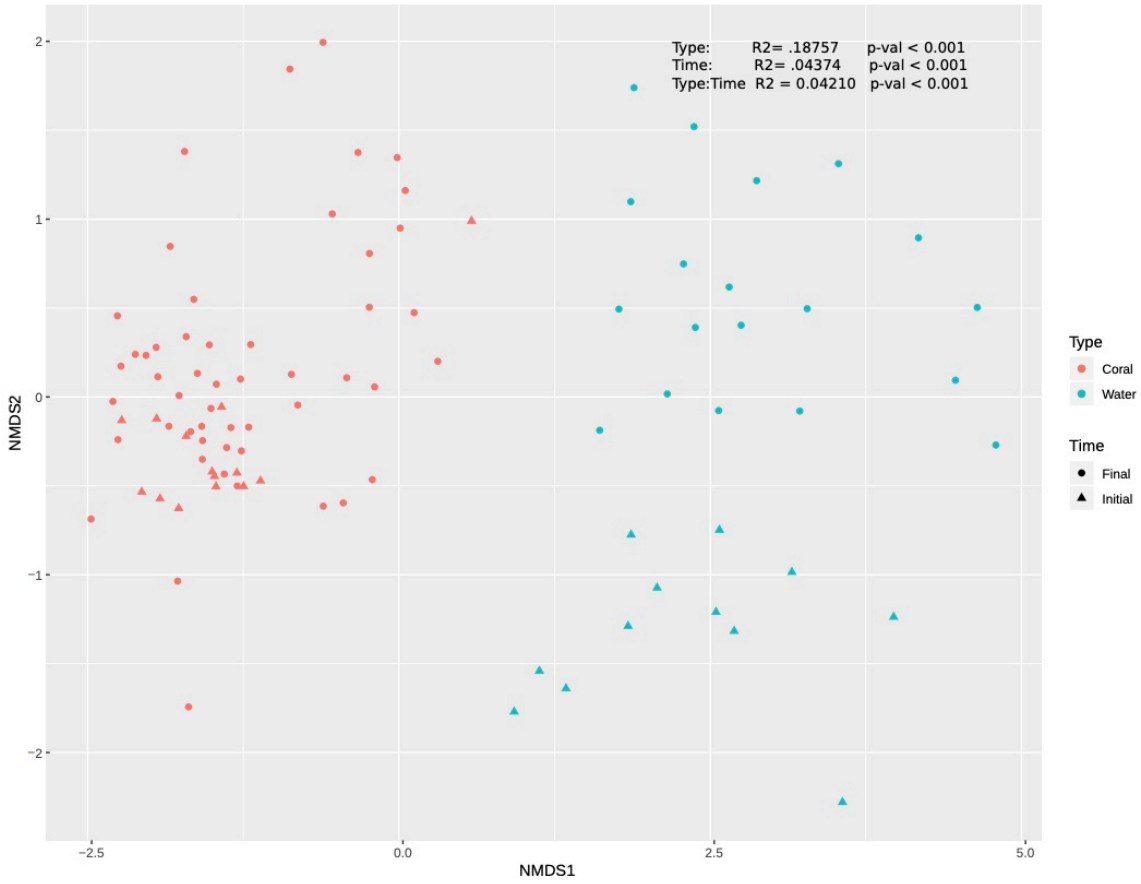

Figure C1: NMDS plot of bacterial microbiome composition (Bray-Curtis dissimilarity).